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Colonization of Porcine Intestine by Enterotoxigenic *Escherichia coli*: Selection of Piliated Forms In Vivo, Adhesion of Piliated Forms to Epithelial Cells In Vitro, and Incidence of a Pilus Antigen Among Porcine Enteropathogenic *E. coli*

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In contrast to K88-positive porcine enterotoxigenic *Escherichia coli* (ETEC), K88-negative porcine ETEC strains did not adhere to isolated intestinal epithelial cells in vitro. However, they did adhere to intestinal epithelium in vivo. Growth of one such ETEC (strain 987) in pig small intestine consistently yielded a greater percentage of piliated cells than did growth in vitro. This increase was demonstrable by electron microscopy, by change in colonial morphology, and by agglutination in specific antisera against the pili of strain 987. In contrast to the stored stock culture (which contained very few piliated cells), richly piliated forms of strain 987 did adhere to isolated intestinal epithelial cells in vitro. A series of porcine *E. coli* strains was tested for agglutinability in antiserum against the pili of strain 987, and several K88-negative ETEC strains were agglutinated. These data are consistent with the hypothesis that pili facilitate intestinal adhesion and colonization by K88-negative ETEC strains.

Colonization of mammalian small intestine by enterotoxigenic *Escherichia coli* (ETEC) is apparently dependent upon the ability of such strains to adhere to villous epithelium in the small intestine (1, 2, 6, 13, 15). There is evidence that this adhesive attribute is conferred by pili or pilus-like structures produced by the ETEC (6, 8, 9, 16). An in vitro system for studying adhesion to intestinal epithelial cells has been developed for porcine ETEC strains that have the pilus-like K88 antigen (14, 18). However, other porcine ETEC strains that lacked the K88 antigen, and non-enterotoxigenic *E. coli* (NETEC), did not adhere to intestinal epithelial cells in vitro. The objectives of the work reported here were: (i) to adopt or develop an in vitro system for the study of the adhesive abilities of porcine ETEC strains that lack K88 antigen; and (ii) to determine the role of pili in the in vitro and in vivo adhesion of one such ETEC strain to intestinal epithelium.

MATERIALS AND METHODS

E. coli. All strains used for in vitro adhesion tests were isolated from pig intestine and are listed in Table 1. The colonizing and adhesive attributes for pig intestine in vivo, as well as enterotoxigenicity, pilus production, and hemagglutinating abilities of these strains, have been reported previously (8, 13). Bacteria were grown and maintained in Trypticase soy broth (TSB) or on Trypticase soy agar (TSA) slants (BBL) and stored at 4°C unless otherwise stated.

The in vitro adhesion test, using isolated intestinal epithelial cells, was performed as described by Wilson and Hohmann (18).

Adherent ETEC and intestinal mucus in vivo. The effect of washing on mucus removal and adhesion of *E. coli* was tested in ligated intestinal loops for strains 431 and 123 and in intact pigs for strain 987. Ligated intestinal loops were created in hysterectomy-derived, colostrum-deprived (HDCD) pigs (20 to 30 h old) as described earlier (2) and inoculated with 10^7 bacteria of the test strains in 1 ml of fresh TSB. Pigs were killed 6 h postexposure. Intragastric inoculation of HDCD pigs to study colonizing abilities of *E. coli* strain 987 was also described before (2, 13). The following procedure was done on intestinal segments from both ligated loops and intact pigs. A 5-cm piece of the intestinal segment was everted onto a plastic pipette and washed in 100 mM Clealand reagent (Baker) with strong stirring (magnetic stirring device) for 10 min to remove mucus (17). The effect of washing on tissue morphology and on viable bacterial counts was studied. For microscopic investigation, samples were fixed in formaldehyde, embedded in paraffin, sectioned, and stained either with Giemsa or with periodic acid-Schiff (PAS) reagent. This washing procedure removed almost all the PAS-positive amorphous mucus from the villous surface so that the microvilli could be

visualized as a PAS-stained thin border (Fig. 1A and B). Each strain was tested in three intestinal segments from three different pigs. For viable bacterial counts, 4 cm of washed and unwashed everted intestine was transferred into cold 0.3% peptone water and homogenized, and viable bacterial counts were determined (13). Association of bacteria with the mucosal epithelium of the intestinal segments was determined by microscopic evaluation of fluorescent-antibody-stained frozen sections as described earlier (2, 13).

Electron microscopy. Samples for transmission electron microscopy were prepared as for the in vitro adhesion test and then processed and stained as described earlier (12). For direct examination of bacteria, negatively stained preparations were used as described (8).

Preparation of antipilus serum. Rabbits were injected intravenously with formalized suspension of a piliated form of *E. coli* strain 987 grown on blood agar, according to the method of Edwards and Ewing (5). The O and K antibodies were removed from the serum by absorption using both an uncapsulated (K^-) pilus-lacking (P^-) and a capsulated (K^+) P^- variant of this strain (8) as described for O and K absorption (5). With this absorbed antipilus serum (anti-987-P), colonies of *E. coli* 987 containing richly piliated (P^{++}) cells were detected by agglutination of bacteria on a glass slide at a serum dilution of 1:10.

Agglutinability of P^{++} cultures in anti-987-P serum was destroyed by heating the cultures for 10 min at 100°C. The anti-987-P serum did not agglutinate strains carrying K88. Neither did it agglutinate colonies of the human enterotoxigenic H-10407 that were agglutinable by the absorbed anticolonizing factor serum kindly sent us by D. J. Evans (6).

Serological survey for incidence of 987 pilus antigen. The *E. coli* strains tested were isolated from pig intestine and maintained on TSA slants at room temperature in the dark for periods of 2 weeks to 10 years. For the purpose of these studies, strains isolated from baby pigs diagnosed as having enteric colibacillosis (11) were classified as enteropathogenic *E. coli* (EEC). Many of them were tested and found to produce enterotoxins. Strains isolated from normal pigs or pigs with diseases other than enteric colibacillosis were classified as nonenteropathogenic *E. coli* (NEEC). For the serological survey, bacteria were inoculated into TSB and incubated at 37°C for 4 to 6 days until a definite pellicle was formed. From this pellicle, 5% sheep blood agar plates were inoculated and, after 24 h of incubation, 5 to 10 colonies (when available, small and transparent) were tested for agglutinability in the anti-987-P serum, using normal rabbit serum as a negative control. Detection of colonies agglutinable in anti-987-P serum required careful inspection of the pellicle-derived blood agar culture. Usually the small, more transparent colonies were agglutinable whereas the larger, less transparent colonies were not.

TABLE 1. *E. coli strains used and their in vitro adhesion to isolated intestinal epithelial cells of pigs*

Strain	Serotype	Designation	Adherent bacteria/cell	
			Jejunal	Ileal
263	O8:K87,88ab:H19	ETEC[a]	16.8	10.1
1351	O101:K30:NM	ETEC	<1.0	<1.0
613	O101:K30:NM	ETEC	<1.0	<1.0
431	O101:K30:NM	ETEC	<1.0	<1.0
I25	O101:K-:NM	ETEC	<1.0	<1.0
987	O9:K103:NM[b]	ETEC	<1.0	<1.0
340	O9:K+:NM	ETEC	<1.0	<1.0
74-5208	O20:K101:NM[b]	ETEC	<1.0	<1.0
381	O20:K+:NM	ETEC	<1.0	<1.0
123	O43:K-:H28	NETEC[c]	<1.0	<1.0
252	O13:K-:H11	NETEC	5.9	5.9
124	O8:K50:H19	NETEC	<1.0	<1.0

[a] Enterotoxigenic for pigs.
[b] Determination of type K by Frits Ørskov and Ida Ørskov, Statens Serum Institute, Copenhagen.
[c] Non-enterotoxigenic for pigs.

RESULTS

In vitro adhesion tests were carried out using isolated epithelial cells from both the ileum and jejunum of three pigs. The K88-negative ETEC did not adhere to any of these cells (Table 1).

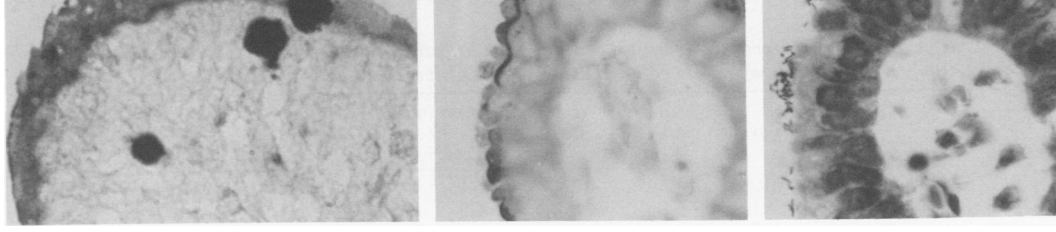

FIG. 1. *(A) Thick PAS-stained mucopolysaccharide layer before washing. Frozen section from a villous tip in the small intestine. PAS, ×300. (B) Frozen section from a villous tip in the small intestine comparable to that in (A). Lack of the PAS-positive mucus layer after washing with 100 mM Clealand reagent. PAS, ×300. (C) Bacteria adherent to the intestinal epithelium of the same villus as in (B) after they were washed with Clealand reagent. Giemsa, ×300.*

Two of the NETEC strains did not adhere either, but one was moderately adherent to both cell types in all three experiments. In contrast, the K88-positive ($K88^+$) strain adhered intensively as described (18).

The fact that the K88-negative ETEC strains used in these experiments had not lost their in vivo adhesive abilities prior to the above in vitro experiments was proved by subsequent intragastric inoculation of HDCD pigs and by inoculation of ligated intestinal loops of HDCD pigs. The K88-negative ETEC colonized and adhered to the ileal epithelium of intragastrically inoculated pigs and to the wall of both ileal and jejunal loops as reported earlier (13).

Some pigs are congenitally resistant to adhesion of $K88^+$ ETEC (2, 14). Experiments were conducted to see if the above failure of K88-negative ETEC to adhere in vitro was caused by the use of cells from pigs resistant to K88-negative ETEC. *E. coli* strains 431, 987, and 74-5208 were inoculated into ligated intestinal loops of two HDCD pigs. After 6 h of incubation, the pigs were killed, and the loops were examined by immunofluorescent microscopy for adherent *E. coli* (association index) (13). The remaining unexposed small intestinal segments from these pigs were used to prepare isolated intestinal epithelial cells, which were used for in vitro adhesion tests with the same strains. All three K88-negative ETEC adhered intensively in the loops of both pigs 6 h postexposure, but none of the *E. coli* strains adhered to the isolated intestinal epithelial cells of the same pigs in vitro. We concluded that the principal strains would not adhere in vitro even when the epithelial cells used came from pigs susceptible to adhesion in vivo.

Subsequently, several trials were made to modify the in vitro adhesion test so that it would demonstrate adhesiveness of the K-88-negative ETEC. Calcium and Mg^{2+} ions, bile salt, sodium deoxycholate, *E. coli* enterotoxins (heat stable and heat labile), and fluid from positive ligated intestinal loops after exposure to ETEC were added to the system, but without any major improvement in the adhesiveness of the K88-negative ETEC over the NETEC strains. In further experiments, the isolated intestinal epithelial cells were replaced by the following cultured mammalian cell lines from the American Type Culture Collection: Y1 (mouse adrenal cell), intestine 407, and HeLa. The K88-carrying strain (used as a positive control) had a tendency to adhere to the above cells, but the K88-negative ETEC were less adherent and indistinguishable from the NETEC strain that was used as a negative control.

In vivo adhesion of K88-negative ETEC and intestinal mucus. Since the K88-negative ETEC did not adhere to epithelial cells in vitro under the above conditions, the following question was raised: Do they adhere to epithelial cells in vivo? Alternatively, are they located in the mucopolysaccharide layer covering the epithelial cells? The vigorous washing of everted intestinal segments from ligated intestinal loops and intact intragastrically exposed pigs with Clealand reagent removed most of the mucopolysaccharide layer from the villous epithelium (Fig. 1A and B). The association indexes of intestinal segments colonized by K88-negative ETEC 987 or 431, however, were not significantly affected; there were still large numbers of bacteria on the washed mucosal surface remaining adherent to the epithelium (Fig. 1c). By using the same washing procedure, the NETEC strain 123 was removed (Table 2).

Differences in colonial morphology and agglutinability in anti-987-P serum. During the experiments reported here, we realized that there were at least two types of colonial morphology of strain 987 on blood agar plates. Some colonies were smaller and more transparent in direct transmitted light than were most of the colonies of that strain when the stock was grown on sheep blood agar at 37°C (Fig. 2). Seven representative colonies from each of these two types were tested in different cultures on 2 separate days. Twenty bacterial cells from each colony were observed under the electron

TABLE 2. *Effect of mucus removal by Clealand reagent on association indexes and on E. coli counts of everted intestinal segments colonized by K88-negative ETEC and NETEC*

E. coli strains	No. of pigs tested	Test system used	Association index[a]			Log_{10} *E. coli*	
			Unwashed	Washed[b]	Significance[c]	Unwashed	Washed
987	4	Intact pig	4.2	4.1	>0.05	8.26	8.37
431	3	Loop	4.8	4.8	>0.05	8.88	8.07
123	3	Loop	3.6	2.0	<0.01	8.24	7.54

[a] Degree of adhesion of bacteria to intestinal epithelium as determined by immunofluorescence microscopy (2, 13).

[b] Washed with 100 mM Clealand reagent for 10 min.

[c] *P* value determined by Student's *t*-test.

microscope, and cells from the same colonies were tested for agglutinability in anti-987-P serum on a glass slide at a serum dilution of 1:10. Based on electron microscopic investigation, 80 to 100% of the cells in these small colonies were P^{++} (Fig. 3). The anti-987-P serum agglutinated bacteria from seven of seven of these small colonies, which contained P^{++} cells of strain 987. In repeated cultures of stock 987 strain on blood agar plates, the P^{++} colonies were consistently in the minority, ranging from about <1 to 5% of the total. Most of the blood agar plate colonies from the stock culture of strain 987 were consistently larger and more opaque than the P^{++} colonies. Electron microscopy revealed that only 0 to 20% of the cells were piliated in these colonies and, when piliated, these had few pili per cell. The anti-987-P serum agglutinated bacteria from none of these seven large colonies, which contained non- or poorly piliated cells ($P^{\pm}$). The above observations on colonial differences for the piliated and nonpiliated phases of *E. coli* are in good agreement with those of Brinton (3).

In vivo selection of piliated forms. Based on the differences in colonial morphology and serological reaction between P^{++} and $P^{\pm}$ forms of *E. coli* 987, we compared the percentage of the P^{++} colonies in six in vitro TSB-grown inocula with that in the population isolated from the ilea of 16 HDCD pigs colonized by *E. coli* 987 16 h postexposure. The results in Table 3 show a 20-fold increase in the percentage of P^{++} colonies in vivo compared with that in vitro.

In vitro adhesiveness of the P^{++} form of *E. coli* 987. We isolated one strain (987 P^{++}) from one of the P^{++} colonies and tested it for in vitro adhesiveness using isolated intestinal epithelial cells (18) along with the stock *E. coli* 987 and with positive (*E. coli* 263) and negative (*E. coli* 123) *E. coli* as control strains. The in vitro adhesiveness of this P^{++} variant of *E. coli* 987 was significantly ($P < 0.01$) higher than that of the parent strain and of the negative control,

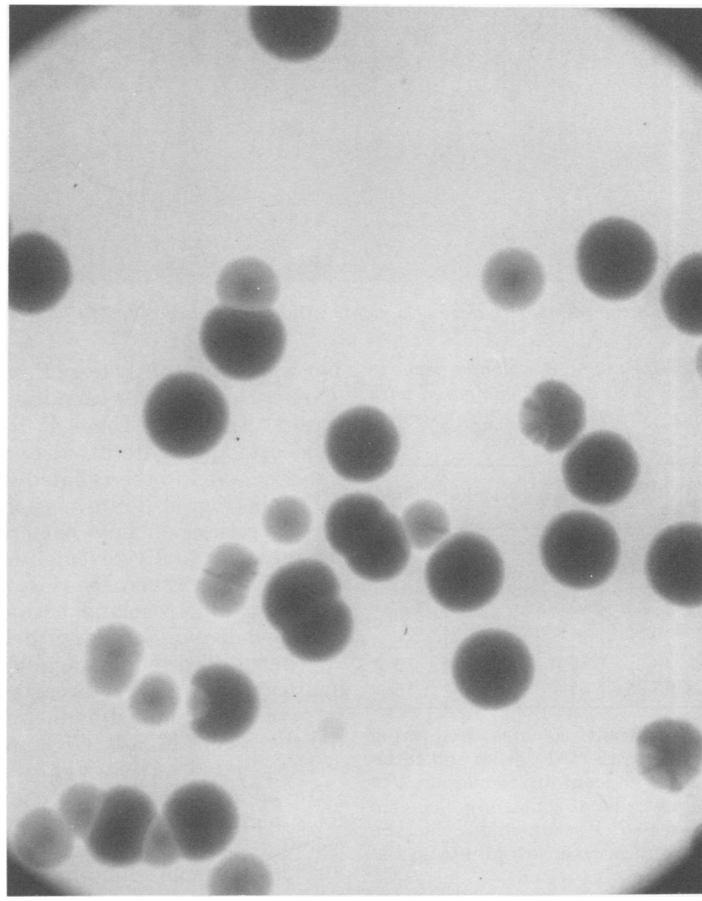

FIG. 2. *Blood agar culture of small P^{++} and large $P^{\pm}$ colonies of E. coli 987 derived from the ileum of an HDCD pig 16 h postexposure.*

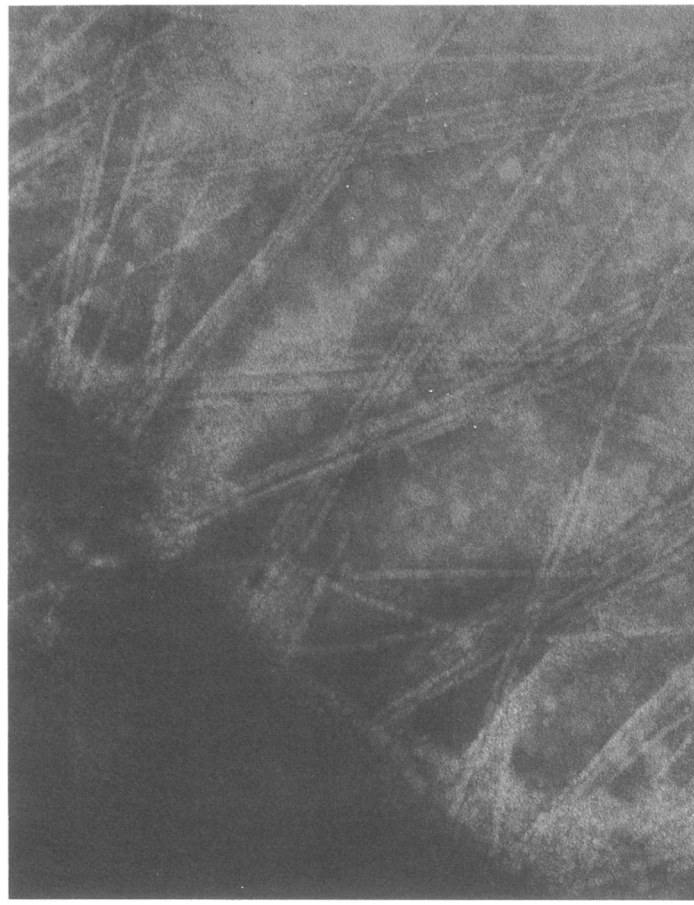

FIG. 3. *Part of the piliated surface of a richly piliated variant of E. coli 987 (987 P++).*

TABLE 3. *Prevalence of P++ and P± colonies in the in vitro grown inoculum of E. coli 987 compared with the population recovered from the small intestine of newborn pigs 16 h postexposure*[a]

Population	P++ small colonies		P± large colonies		No. of tests[b]
	%	No. of colonies agglutinable/no. tested[c]	%	No. of colonies agglutinable/no. tested[c]	
Inoculated	2.4	9/9	97.6	0/9	6
Recovered	48.3	95/96	51.7	1/98	16

[a] P++, Small, translucent colonies containing bacteria agglutinable in anti-987-P serum and of the type shown previously to contain numerous P++ cells. P±, Large opaque colonies containing bacteria not agglutinable in anti-987-P serum and of the type shown previously to contain no or few piliated cells.

[b] Number of inocula or pigs tested.

[c] Number of colonies with bacteria agglutinable in anti-987-P serum over number tested.

but was significantly less ($P < 0.01$) than adhesiveness of the K88-carrying positive control strain (Table 4). These P++ variants of 987 tended to aggregate in vitro, and these aggregates adhered to the isolated intestinal epithelial cells (Fig. 4). Transmission electron microscopic sections of these bacteria-epithelial cell aggregates usually revealed large numbers of bacteria with several surface appendages, resembling pili, reaching between the bacteria and the microvilli of the epithelial cells (Fig. 5).

When tested for hemagglutinating abilities against guinea pig erythrocytes (8, 10), the P++ form of 987 remained nonhemagglutinating as did the parent *E. coli* 987 strain (Table 4).

The piliated variant of 987 (987 P++) lost its in vitro adhesiveness during laboratory storage for 2 months at 4°C as well as its characteristic colonial morphology and agglutinability in anti-987-P serum. Subsequently, an additional three P++ variants (987 P++-1, -2, and -3) were isolated from the ileum of *E. coli* 987-

colonized newborn pigs based on colonial morphology and agglutinability in anti-987-P serum. The fresh piliated isolates contained a high (32.5 to 98.9) percentage of small colonies with agglutinable cells, whereas only a very low (<1.0 to 2.6) percentage of these colonies was found in the cultures of 987 and 987 P^{++}. Variants 1, 2, and 3 were tested for in vitro adhesiveness in three experiments parallel with the once richly piliated isolate (987 P^{++}). All three fresh isolates adhered significantly ($P < 0.01$) more than did the negative control NETEC or the parent *E. coli* 987. The isolate, 987 P^{++}, was indistinguishable from the negative control or parental *E. coli* 987 strain (Table 5). In addition, the parental *E. coli* 987 and once richly piliated 987 P^{++} forms were different from the fresh, P^{++} isolates (987 P^{++}-1, -2, and -3) in terms of colonial morphology.

Incidence of 987 pilus antigen among porcine EEC strains. Using the absorbed anti-987-P serum, we tested 119 EEC and 54 NEEC strains for agglutinability in this serum by slide agglutination. As the results of Table 6 show, 50% of the O9 and 14% of the O20 EEC strains contained colonies that were agglutinated by the anti-987-P serum, whereas none of the EEC from serogroup O101 or from serogroups O8, O138, O139, or O141 did so. In addition, all the NEEC strains tested were negative.

DISCUSSION

The in vitro adhesion tests confirmed the report of Wilson and Hohmann (18) that K88-carrying *E. coli* strains adhere, whereas K88-negative ETEC and NETEC do not. Trials to modify the test so that it could be used to demonstrate in vitro adhesiveness of stock cultures of K88-negative ETEC were unsuccessful. Based on the observation that K88-negative EEC colonized the ileum but not the jejunum in vivo (13), we expected some adhesion to ileal cells in vitro; however, this was not the case. The data indicate that the lack of in vitro adhesiveness by K88-negative EEC was not due to the loss of in vivo colonizing and adhesive abilities of these strains or to the resistance of epithelial cells used. Furthermore, as the results of Table 2 show, the principal K88-nega-

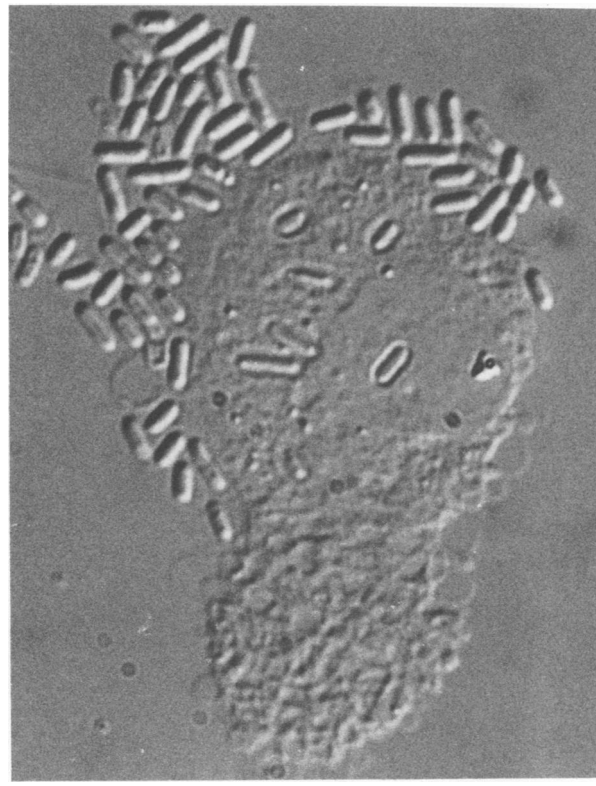

FIG. 4. *Bacteria of a richly piliated variant of E. coli 987 (987 P^{++}) adherent to an isolated intestinal epithelial cell in vitro.*

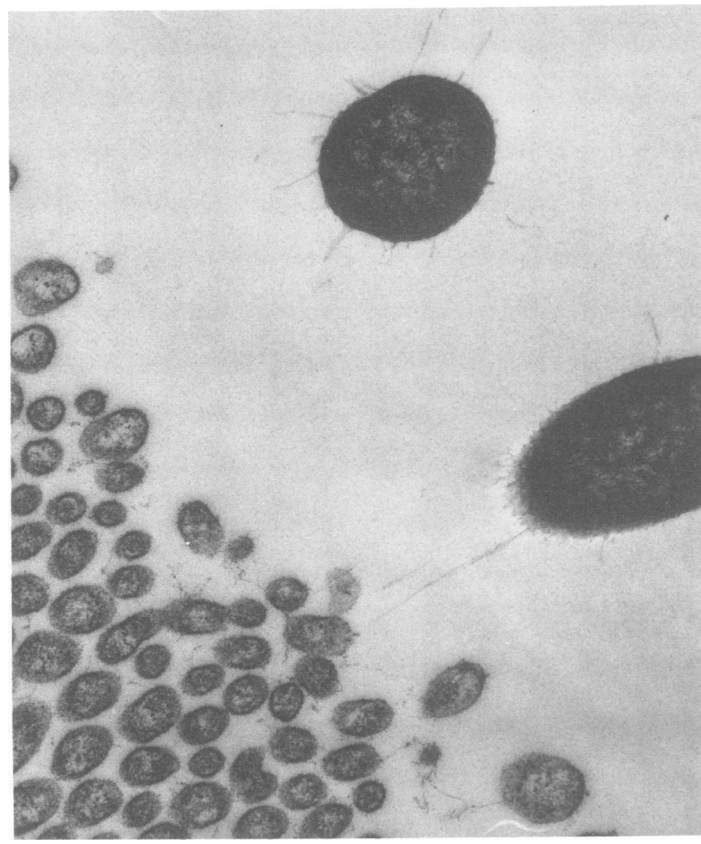

FIG. 5. *Richly piliated variant of E. coli 987 (987 P^{++}) with surface appendages extending to the microvilli of isolated intestinal epithelial cell in vitro. Transmission electron micrograph of an ultrathin section.*

TABLE 4. *In vitro adhesion and hemagglutination of E. coli 987: its P^{++} form in comparison with positive and negative control E. coli strains*

E. coli strains	Designation	Hemagglutination[a] of guinea pig erythrocytes	Adherent bacteria/isolated intestinal epithelial cells[b]	
			Mean	Range
263	ETEC, K88$^+$	MR	17.6	15.3–18.7
123	NETEC	MS	1.7	1.0–4.2
987	ETEC, K88$^-$	–	2.1	1.0–5.1
987 P^{++}	ETEC, K88$^-$	–	6.0	3.0–9.7

[a] MR, Mannose-resistant hemagglutination; MS, mannose-sensitive hemagglutination; –, no hemagglutination.

[b] Results of duplicate experiments on three different days.

tive ETEC were strongly attached to the epithelial cells in vivo and remained there in spite of removal of the surface mucus layer.

The differences between in vitro and in vivo adhesiveness of the *E. coli* 987 strain could be explained by the difference in pilus production under these two conditions. Data in Table 3 indicate that growth in pig intestine either selects for or promotes the development of piliated forms. A relatively higher growth rate of piliated than of nonpiliated forms in vivo could be the result of limited oxygen present in the intestine, as suggested by Brinton (4). It is logical to assume that an essential part of the adherent bacterial layer formed on the small intestinal epithelium in vivo is composed of P^{++} forms of the bacteria. There is ultrastructural evidence to support that assumption (12). P^{++} forms had increased in vitro adhesiveness to isolated intestinal epithelial cells, and the surface appendages reaching from the bacteria towards the isolated epithelial cells can be best interpreted as pili (Fig. 5).

However, the P^{++} colonies, isolated from colonized ilea of newborn pigs, tended to lose their characteristic colonial morphology during laboratory storage in TSB or on TSA and during

TABLE 5. *In vitro adhesion of piliated forms of E. coli 987: comparison of stored cultures with fresh isolates*

Status of cultures	*E. coli* strains	Adherent bacteria/cell		Small (P^{++}) colonies (%)	
		Mean	Standard error	Mean	Range
Stored	263	19.24	1.37	NT[a]	
Stored	123	0.17	0.18	NT	
Stored	987	0.11	0.08	<1.0	
Stored	987 P^{++}[b]	0.51	0.91	2.1	1.8–2.6
Stored	987 P^{++}[c]	1.89	1.70	5.7	4.6–6.7
Fresh	987 P^{++}-1	5.28	2.97	98.4	98.0–98.9
Fresh	987 P^{++}-2	3.73	1.57	38.2	32.5–49.5
Fresh	987 P^{++}-3	2.90	1.90	52.9	42.6–66.7

[a] Not tested.

[b] Once richly piliated variant of *E. coli* 987 stored on TSA slant for 2 months.

[c] Once richly piliated variant of *E. coli* 987 stored in TSB for 2 months.

TABLE 6. *Results of serological survey among porcine E. coli strains with anti-987-P serum*

Serogroup	No. of strains agglutinable/no. tested	
	Enteropathogenic	Non-enteropathogenic
O9	11/22	0/9
O20	3/21	0/12
O101	0/16	0/2
Others	0/60	0/31

subsequent cultures on sheep blood agar, and they tended to revert back to $P^{\pm}$ form. The loss of in vitro adhesiveness of the strain 987 P^{++} was probably due to the loss of its piliated form during 2 months of laboratory storage as indicated by the low percentage of P^{++} colonies in blood agar cultures of this strain compared with the adherent, fresh isolates 987 P^{++}-1, -2, and -3 (Table 5). This line of evidence, together with the lack of in vivo colonizing and adhesive abilities of P^{-} variants of *E. coli* 987 (8), indicates the importance of pili in the virulence of this strain.

It could be that under circumstances similar to those of 987 P^{++}, NETEC strains would also form large numbers of pili and adhere in vitro. In fact, one of our NETEC strains (*E. coli* 252) was moderately adhesive (Table 1). In earlier studies, however, when HDCD pigs were intragastrically inoculated with three different NETEC strains, none of them (including strain 252) colonized or adhered to the small intestinal epithelium (13). These NETEC strains were all piliated (8), but none of them agglutinated with the absorbed anti-987-P serum. Furthermore, all three NEEC strains showed mannose-sensitive hemagglutination of guinea pig erythrocytes, indicating a different kind of pilus from that of 987. Functional differences among pili could be one explanation for the lack of in vivo adhesiveness by these NETEC strains. On the other hand, pili may be necessary, but not the only factor, for effective colonization of the small intestine.

The fact that several EEC strains from serogroups O9 and O20 reacted with the anti-987-P serum leads us to suggest that this pilus antigen is not confined to strain 987. Further studies are needed to determine the degree of antigenic relatedness, anatomic nature, and functional significance of these antigens on the other strains.

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