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Capsule Reduces Adherence of Enterotoxigenic *Escherichia coli* to Isolated Intestinal Epithelial Cells of Pigs

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Previous reports have demonstrated that heat-stable (A-type) capsule on piliated enterotoxigenic *Escherichia coli* enhances colonization of enterotoxigenic *E. coli* in the small intestine and enhances virulence of enterotoxigenic *E. coli*. In this report, four encapsulated enterotoxigenic *E. coli* strains and one encapsulated nonenterotoxigenic strain of *E. coli* and their nonencapsulated mutants were tested for adhesion to isolated intestinal epithelial cells or brush borders from neonatal pigs. The enterotoxigenic *E. coli* also expressed the K99 pilus antigen. The nonencapsulated mutants of the four enterotoxigenic *E. coli* adhered in higher numbers than did the encapsulated parental strains. Both the encapsulated and nonencapsulated forms of enterotoxigenic *E. coli* 431 grown at 18°C (K99 production suppressed) adhered poorly to the isolated cells. The nonenterotoxigenic *E. coli* 1793 which does not express K99 antigen also adhered poorly in both encapsulated and nonencapsulated forms. Fab fragments of anticapsular immunoglobulin G failed to block the effect of capsule on adherence of strain 431. The results indicated that K99 was the principal mediator of in vitro adhesion of the enterotoxigenic *E. coli* strains and that capsule impedes the in vitro adhesion. They also suggested that the capsular enhancement of colonization by such strains in vivo probably is by some mechanism other than enhanced adhesion to epithelium.

Colonization (proliferation to high numbers) by enterotoxigenic *Escherichia coli* (ETEC) in the small intestine of animals contributes to the virulence of ETEC. Several in vivo and in vitro studies have shown that specific filamentous, protein appendages called pili or fimbriae which are on the surface of ETEC facilitate adhesion of ETEC to small intestinal mucosa and facilitate colonization by ETEC in the small intestine (9, 19, 20, 24, 26). Pilus antigens associated with ETEC of pigs are currently named K88, K99, 987P, and F41. Ørskov and Ørskov have proposed a pilus antigen nomenclature in which K88, K99, and 987P are called F4, F5, and F6, respectively (22). K99 and F41 are also associated with ETEC of calves and lambs.

Bacterial polysaccharide capsule is associated with bacterial colonization of many inert and biological surfaces (3). Many strains of ETEC from animals produce capsules (K antigens) of the A type (heat stable, mucoid, high molecular weight, and low electrophoretic mobility toward the anode) (21). These A-type capsules tend to enhance the colonizing ability of the strains that produce them (7, 20, 26), in that piliated acapsular mutants of many strains colonize the small intestine less intensively than do the encapsulated parental strains. Capsular polysaccharide is closely associated with the ileal microvilli and glycocalyx of calves colonized with encapsulated ETEC (1, 2).

Recent reports indicated that an encapsulated ETEC strain was more virulent in suckling neonatal pigs than was its acapsular mutant and that capsular antigen was shown to protect against challenge with the encapsulated strain in vaccines given to swine (16; P. L. Runnels and H. W. Moon, *Proceedings of the 19th United States-Japan Joint Cholera Conference*, in press). Possible mechanisms by which polysaccharide capsule enhances colonization and virulence, suggested by Costerton et al. (3), included mediation of adherence (possibly in conjunction with pili), stabilization of microcolonies, protection from soluble products in the intestinal lumen, protection from secreted products of the immune system, and resistance to phagocytosis.

Adherence of ETEC to isolated membranes, cells, or tissue sections of the small intestinal mucosa is an in vitro correlate to in vivo colonization by ETEC (5, 9, 24). This system has been used to study pilus-mediated adherence of ETEC. In this report, several strains of encapsulated piliated ($K99^+$) *E. coli* and their acapsular mutants were used to determine whether A-type capsule enhances pilus-mediated adhesion to isolated intestinal epithelial cells. The data do not support that hypothesis.

MATERIALS AND METHODS

***E. coli* strains.** The encapsulated parental strains are listed in Table 1. Acapsular mutants were selected from nonmucoid variants of isolated colonies of the parent strains grown on 5% sheep blood agar for 48 to 72 h at 37°C unless stated otherwise (27). Acapsular mutant I26 previously had been selected from strain 431 (20). Strains used for the adhesion assays were grown on minca agar (minus glucose plus IsoVitaleX [BBL Microbiology Systems, Cockeysville, Md.]), which enhances expression of K99 pili (6). The bacteria were suspended from agar in Krebs-Henseleit (KH) buffer, pH 7.4 (24), with 1% (wt/vol) D-mannose (KHM) added. The KHM-suspended cultures were adjusted to about 2×10^9 *E. coli* per ml as determined spectrophotometrically. The actual concentration of viable *E. coli* was determined by a spiral dilution method (Spiral Systems, Inc., Cincinnati, Ohio). To study the effect of concentration, bacteria of strain 431 and its acapsular mutant, I26, were varied over a 15-fold range of concentration. Presence of capsule was determined by colonial morphology on agar (27), agglutination of resuspended cultures in anti-capsule (K antigen) specific antiserum, and failure to agglutinate in anti-cell wall (O antigen) specific antiserum. Absence of capsule was demonstrated by nonmucoid colonial morphology, agglutination of resuspended culture in cell wall specific antiserum, and failure to agglutinate in capsule specific antiser-

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TABLE 1. Some characteristics of encapsulated parent strains of *E. coli* used in the in vitro adhesion assays

Strain no.	Serotype[a]	Enterotoxin (STa)	Species of origin[b]	Reference or source
431	O101:K30,99:NM	+	Swine	Moon (15, 17)
1793	O101:K30:H1	0	?	Moon (16)
B44	O9:K30,99:NM	+	Bovine	Smith (25)
Troyer	O9:K35,99:NM	+	Swine	Kohler (12)
77-0096	O101:K28,99:NM	+	?	Wilson[c]

[a] NM, Nonmotile.
[b] ?, Not recorded.
[c] *E. coli* Reference Center, Pennsylvania State University, State College, Pa.

um. The antisera were obtained from Richard Wilson (*E. coli* Reference Center, Pennsylvania State University, State College, Pa.). All strains were tested for expression of K99 antigen by slide agglutination with specific antiserum (18).

Intestinal epithelial cells. Isolated intestinal epithelial cells (enterocytes) were prepared by a modification of a previously described method (28). Suckling pigs (2 to 5 days old) from 3 different herds were sacrificed, and their small intestines were removed and stored for 1 h in KH buffer at 4°C. Segments were inverted onto stainless-steel rods (diameter, 3/16 in. [0.48 cm]) and shaken at high frequency, low amplitude in KH buffer for 10 min each. The entire small intestine was used. The resultant cell suspensions were washed three times by low-speed (200 × *g*) centrifugation, followed by resuspension in KHM buffer. The final suspension was adjusted to 2×10^6 enterocytes per ml, as determined by counting enterocytes in a hemacytometer chamber. The suspensions were used without further washing. Fresh enterocytes were isolated each week.

In vitro adhesion assay. Equal volumes (0.5 ml) of enterocyte suspension and *E. coli* suspension were mixed and then incubated at 37°C with shaking for 1 h. The mixture was washed twice by centrifugation at 200 × *g* and resuspended in ca. 1 ml of KHM buffer. The final pellet was suspended in a minimal (ca. 0.1 ml) amount of KHM buffer. The suspensions were coded, and bacteria adherent to the first 20 enterocytes or brush borders were counted microscopically, using ×1,000 magnification and differential interference contrast optics, by each of two observers (total counted, 40 cells). An upper counting limit of 70 bacteria per enterocyte was set arbitrarily. The results were decoded, and the mean ± standard deviation was calculated for each 20-cell sample in a replicate. Three such replicate assays were done on consecutive days, using freshly grown bacteria each day.

Preparation of Fab fragments. A protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) liquid chromatography column was used according to the recommendations of the supplier to isolate immunoglobulin G from the serum. Fab fragments of anti-K30 and anti-K88 antisera were prepared by the method of Mage (13). Undigested immunoglobulin G and Fc fragments were removed from the mixture by passing it over the protein A-Sepharose CL-4B column. The Fab solution was concentrated 10-fold with a Minicon B15 concentrator (Amicon Corp., Danvers, Mass.). After concentration, there was a solution of ca. 1.4 mg of protein per ml.

RESULTS

Nonencapsulated ETEC consistently adhered to enterocytes in higher numbers than did the respective encapsulated parental strains (Fig. 1). Encapsulated or nonencapsulated forms of strain 431 grown at 18°C adhered poorly to the isolated epithelial cells (Fig. 1). Strain 1793, which is nonenterotoxigenic and does not produce any known ETEC-associated pili, adhered poorly in both the encapsulated and nonencapsulated forms.

Anti-K30 Fab fragments mixed with strain 431 did not interfere with adherence (Fig. 2). The fragments did not cause agglutination of strain 431 *E. coli*. The presence of Fab on the surface of the strain 431 *E. coli* was demonstrated by agglutinating the Fab-treated *E. coli* with anti-Fab serum. Similarly, Fab-treated strain 431 *E. coli* also were agglutinated in anti-K30 serum.

The effect of bacterial concentration on adherence was tested (Fig. 3). The increased adherence with increasing concentration of *E. coli* is roughly parallel for the encapsulated strain 431 and its acapsular mutant strain I26. At equivalent concentrations, the acapsular mutant adhered about three times greater than its encapsulated parent strain.

There was variability (not obvious in Fig. 1) in the number of *E. coli* adherent to epithelial cells of pigs from different litters and occasionally of pigs from the same litter. This phenomenon is exemplified in Fig. 4, which depicts the results of an experiment in which epithelial cells from pigs in two litters were compared in one series of adhesion assays. The adherence of both encapsulated strain 431 and its acapsular mutant I26 was greater to cells of pigs in litter A (pigs 50 and 51) than to cells of pigs in litter B (pigs 52 and 53).

DISCUSSION

The data presented did not support the hypothesis that polysaccharide capsule (A type) enhanced pilus-mediated adhesion of ETEC to isolated intestinal epithelial cells. We previously observed that a nonencapsulated strain of *E. coli* adhered better in vitro than did an encapsulated strain in a system similar to that used here (23). However, the strains had been isolated from different species, which limited the significance of the observation. In this study, four ETEC strains were selected on the basis of capsular antigen, cell wall antigen, and K99 pilus antigen. All four strains, representing three different A-type capsular antigens, behaved similarly in the adhesion assays; that is, the acapsular

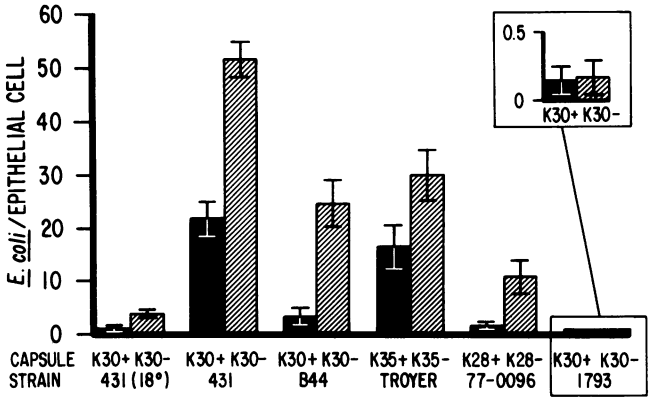

FIG. 1. Nonencapsulated ETEC adhered to isolated enterocytes in higher numbers than did the respective encapsulated parental strains. All strains except strain 1793 produced K99 pilus antigen (strain 431 grown at 18°C did not express the K99 antigen). The bars represent the mean ± standard error. Triplicate assays were done on the cells from each pig (8 pigs for strain 431, 10 pigs for strains B44 and Troyer, and 6 pigs for strains 77-0096 and 1793).

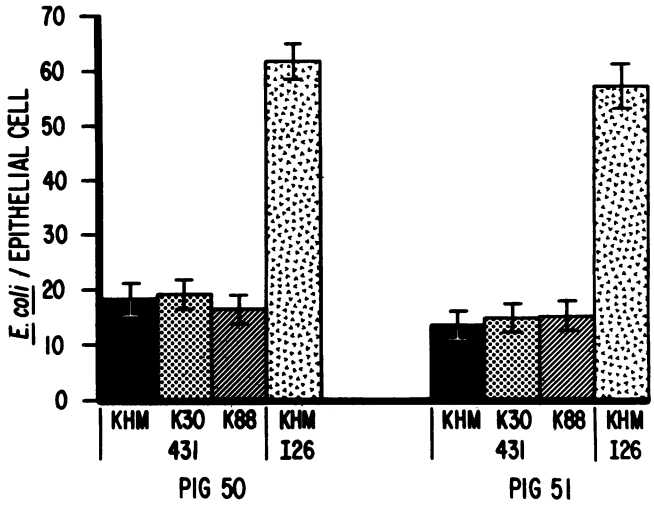

FIG. 2. There was no effect of anti-K30 Fab fragments on adhesion of strain 431 to isolated intestinal epithelial cells. K30 and K88 are the antigens against which the Fab fragments were directed. Anti-K88 Fab was used as a control for nonspecific binding. The assays were done in triplicate for each pig. Only KHM buffer was used with the acapsular mutant strain I26. The bars represent the mean ± standard error for triplicate assays.

mutant adhered in higher numbers than did the encapsulated parent strain. A previous report indicated that the K103 capsule of a 987P piliated *E. coli* did not enhance in vitro adhesion to porcine epithelial cells (9). In contrast to the results with $K99^+$ ETEC reported here, there was no evidence that capsule reduced in vitro adherence of $987P^+$ ETEC (9). The 987P pili are structurally, chemically, and antigenically distinct from K99 pili (4, 8, 10, 11) and are likely to interact differently with surrounding capsule and epithelial cells.

Anti-K30 Fab did not affect adherence of the encapsulated 431 ($K30^+$) ETEC. The failure of anti-K30 Fab to block adhesion suggests that K30 is not mediating adhesion and is consistent with observations that anti-pilus Fab inhibited adherence of homologously piliated *E. coli*, whereas Fab against non-pilus antigens had no effect on adhesion (9). If capsule sterically hindered the adherence, the addition of Fab fragments would have been expected to further contribute to hindrance or possibly to have no additional effect. It is possible that in this case too little anti-K30 Fab was available either to block or to contribute to the effect of capsule, since *E. coli* coated with the anti-K30 Fab still was agglutinated with anti-K30 serum.

One possible explanation for the reduced adherence of the encapsulated strain 431 is that the space occupied by the capsule nonspecifically blocked cell receptors which would otherwise be exposed for attachment by piliated bacteria. That does not seem to be true, since varying the concentration of strain 431 *E. coli* (Fig. 3) used in the assay showed that about twofold more bacteria adhered at the highest concentration than at the concentration routinely used. This indicated that the brush borders were not saturated with encapsulated *E. coli* at the lower concentration. In contrast, the numbers of adherent bacteria of the acapsular mutant I26 were near the upper counting limit for the assay at concentrations routinely used. It seems likely that in vitro the capsule could mask many of the K99 pili on piliated, encapsulated strains, much as it masks the O antigen during serological characterization. This is supported by the observation of slow, diffuse agglutination of encapsulated, piliated strains in anti-K99 serum compared with rapid, flocculent agglutination of nonencapsulated, piliated mutants. Furthermore, electron micrographs have shown that capsule and pili occur in similar regions over the bacterial cell surface (1).

Two possible sources of variation in adherence which occurred between litters (exemplified in Fig. 4) are differences in passively acquired immunity and differences in congenital or genetic resistance to K99-mediated adherence. The enterocytes were from suckling pigs whose dams had not been vaccinated against *E. coli*. However, their immunity to ETEC is unknown. In vitro adherence of piliated *E. coli* can be inhibited by adding specific antipilus antiserum to the

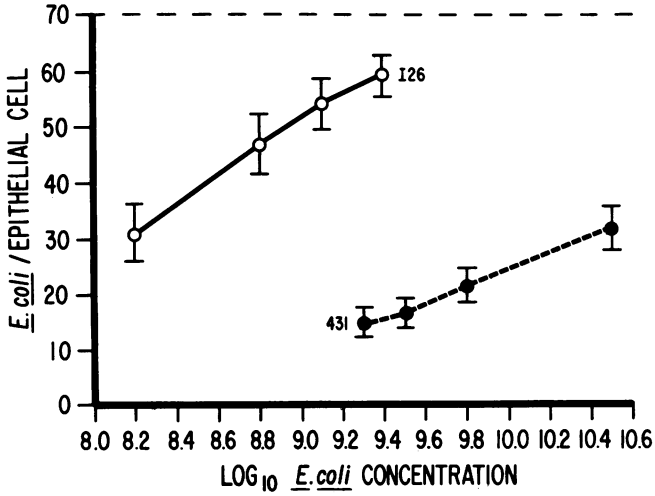

FIG. 3. There was an effect of concentration on adherence of strain 431 and its acapsular mutant, strain I26. The data were pooled from triplicate assays in which enterocytes from four pigs were used. The standard error of the mean bacterial concentrations shown was ±0.1 at all concentrations. The T-bars represent the standard error of the triplicate assays. The dashed horizontal line at 70 *E. coli* per epithelial cell indicates the arbitrary upper counting limit.

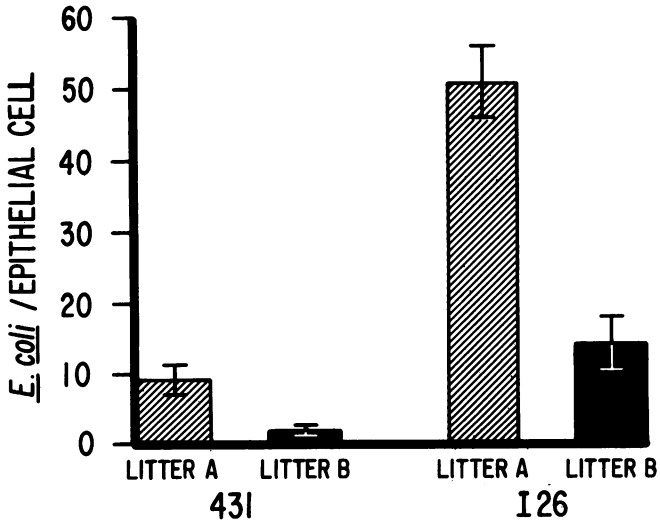

FIG. 4. There was variation in the number of bacteria adherent to epithelial cells of pigs from different sources. Litters A and B were from different herds, and both litters had suckled unvaccinated dams. The bars represent the mean ± standard error of triplicate assays in which two pigs per liter were used.

E. coli suspension (5, 9). Although passive immunity to pilus antigen inhibits the effects of in vivo challenge with piliated ETEC (14), the effect of passive immunity of the source pigs on in vitro adherence of ETEC has not been reported. In contrast, genetic resistance of pigs to $K88^+$ ETEC is apparent both as lack of response to in vivo challenge and as decreased adherence in vitro (24). If that phenomenon occurs with $K99^+$ ETEC, the effect is not as marked as it is with $K88^+$ ETEC. There is variation among neonatal pigs in their response to challenge with ETEC strain 431. In one study, 50% of the challenged pigs were resistant to lethal effects of strain 431 (14), and more recently, only 26% were resistant to similar challenge (16). The observations presented here are consistent with, but do not prove, congenital or genetic resistance to K99-mediated adherence.

The data in this report suggest that the mode by which capsule enhances colonization is not through enhanced adherence to intestinal mucosa. Although the in vitro adhesion assay correlates with in vivo colonization in many cases, it is an artificial system isolated from normal physiological effects, such as gut motility, the digestive milieu, and secretory and cellular immune processes. Furthermore, in vitro adhesion which is assayed after 1 h of incubation is likely to represent a small fraction of the events occurring during ETEC colonization of the small intestine, which is usually assayed 18 to 24 h after oral inoculation. Efforts to define the mechanisms of adherence as they relate to colonization and the roles of capsules in those events will be made with in vivo models.

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