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Immunization of Suckling Pigs Against Enterotoxigenic *Escherichia coli*-Induced Diarrheal Disease by Vaccinating Dams with Purified 987 or K99 Pili: Protection Correlates with Pilus Homology of Vaccine and Challenge

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Pregnant gilts were vaccinated with purified strain 987 pili (987P), strain K99 pili, or a saline-formaldehyde control. Suckling pigs born to vaccinated gilts were allowed to consume colostrum and were then challenged intragastrically with one of three enterotoxigenic *Escherichia coli* strains: 987 (O9:K103, 987P:NM), 74-5208 (O20:K101, 987P:NM), or 431 (O101:K30, 99:NM). In litters where the dam was vaccinated with the same pilus as that possessed by the challenge organism, the incidence and duration of diarrhea and the degree of intestinal colonization (either duration or extent) were less than those of the other vaccine groups. Surviving pigs in the homologous vaccine groups also had better weight gains than pigs in the other vaccine groups. These experiments extend and confirm previous reports that vaccination of the dam with purified pili confers protection to neonatal suckling pigs against diarrheal disease caused by enterotoxigenic *E. coli* strains that possess the same pili. Protection did not extend to enterotoxigenic strains possessing different pili.

Pili are nonflagellar, hairlike appendages of bacteria, consisting of assemblies of identical protein subunits (3, 4, 8, 11). Sex pili are required for conjugal DNA transfer and for pilus phage nucleic acid entry (5), and somatic pili, more numerous and widely distributed than sex pili, are associated with the colonization of natural environments (4, 6, 23, 26, 27). Somatic pili are ubiquitous on both pathogenic and nonpathogenic bacteria. However, there is evidence to support the hypothesis that they are among the required factors for pathogenicity (6, 7, 14). The expression of somatic pili is phenotypically controlled by a system termed piliation phase variation (3, 6, 8, 29). The piliated phases of bacteria have a number of properties (the piliated-phase syndrome) that differ from their nonpiliated phases (6). Some of these properties, such as adhesion to mammalian epithelial cells, surface translocation, enhanced growth in limiting oxygen concentrations, and tight colonial association of bacterial growth, appear to adapt the piliated phase particularly well for the colonization of vertebrate epithelium.

Depending upon pilus type, the piliated or nonpiliated phase can be maintained in the lab by cloning since their colonies on agar have different forms (3, 4, 6, 7, 23, 25). The piliated phases of some bacteria are more pathogenic than their nonpiliated phases (6, 7, 16, 19, 26, 27). The ability to clone for piliation has also permitted the growth, concentration, and purification of somatic pili in quantities sufficient for characterization and immunization (4, 7, 11, 13). Pilus antibodies induced by the parenteral administration of purified pili have provided protection against experimental infection by *Neisseria gonorrhoeae, Pseudomonas aeruginosa,* and enterotoxigenic *Escherichia coli* (ETEC) when the pili of the challenge strains were the same as the immunizing pili (6, 7, 18, 24). It has been postulated that neutralization of functions of the piliated phase by specific pilus antibodies is responsible for the protective effect of pilus vaccines (6, 7, 18, 24).

ETEC cause diarrheal disease. To do this, they must colonize the small intestine of the host. Previous investigations have indicated that ETEC colonize small intestine by adhering to the epithelium (1, 2, 14, 17, 22). Somatic pili of porcine ETEC known to be associated with intestinal colonization are: 987P (11, 14, 16, 23), K88 (12, 17, 26, 28), and K99 (13, 14, 20, 25, 27). Vaccination of pregnant gilts with K88 conferred protection to suckling pigs challenged orally with a $K88^+$ ETEC strain (18), and vaccination

of pregnant gilts with 987 pili conferred protection to suckling pigs challenged with a 987P$^+$ strain (24).

The objectives of this study were: (i) to confirm the observation that dams vaccinated with 987P conferred protection to suckling pigs against diarrheal disease after challenge with strain 987; (ii) to determine if protection conferred by 987P extends to pigs challenged with an ETEC strain with O and K serogroups different from 987 but with the homologous pili; (iii) to determine if another pilus, K99, confers protection against diarrheal disease after challenge with an ETEC possessing the K99 pilus; and (iv) to determine if vaccination with pili protects against diarrheal disease after challenge with ETEC possessing serologically different pili.

This report is one in a series (11, 14, 24) describing the results of a cooperative program between the National Animal Disease Center and the University of Pittsburgh to evaluate *E. coli* pilus vaccines in animals. The results of this report were presented in summary form at the XIIIth U.S.-Japan Conference on Cholera in Atlanta, Ga., 19 to 21 September 1977 (15). The results in these reports, taken together with others, support the hypothesis that the serological specificity of pili is correlated with the specificity of immunity, intestinal colonization, in vivo epithelial adhesion, and in vitro epithelial adhesion.

ETEC also cause diarrheal disease in humans (10), calves, and lambs. Somatic pili, serologically related to type 1 pili of *E. coli* (3, 8, 11), have been found on about 40% of ETEC of human origin (6, 7; P. Gemski and C. C. Brinton, unpublished data; M. Levine and V. Daya, personal communication). Pili of the type 1 family are found on piliated phases of *E. coli* H10407 and *E. coli* B7A (7), both of which have caused diarrheal disease in experimentally exposed humans (19a). K99 is common among calf and lamb ETEC (25). The results reported here for pigs further support the need to evaluate type 1 pilus vaccines for the control of human ETEC infections (6, 11) and K99 vaccines for ETEC infections in calves and lambs.

MATERIALS AND METHODS

***E. coli* challenge strains and inocula.** The three challenge strains of ETEC used were 987 (O9:K103, 987P:NM), 74-5208 (O20:K101, 987P:NM), and 431 (O101:K30, K99:NM). All three strains were originally isolated from the intestines of pigs with diarrheal disease, and all produce heat-stable, but not heat-labile, enterotoxin (pig intestinal loop and infant mouse positive, adrenal cell negative; 9, 22, 30). Strains 987 and 74-5208 both undergo phase variations of piliation (3, 6, 11, 23, 24). Colonies of piliated bacteria of each strain were identified as previously described (11, 13, 23, 24). A piliated-phase colony of 987 bacteria (small colony containing cells agglutinable by monospecific 987P antiserum) and a piliated colony of 431 (colony containing cells agglutinable by monospecific K99 antiserum) were each picked from blood agar plates inoculated into separate 10-ml tubes of Trypticase soy broth (TSB) and incubated overnight without shaking at 37°C. Both strains were subcultured (1:500) in 500 ml of fresh TSB and incubated for 18 to 22 h at 37°C without shaking. For the preparation of 74-5208, 500 ml of TSB was inoculated with a portion of a piliated-phase colony of 74-5208 (small colony containing cells agglutinable by monospecific 987P antiserum) and incubated as above. After growth, the bacteria were harvested by centrifugation at 7,000 × *g* for 10 min and resuspended in half-strength TSB containing 10% glycerol to the following concentrations: 987 = 2.5 × 10^8/ml (97% piliated phase); 74-5208 = 5 × 10^9/ml (1% piliated phase); 431 = 5 × 10^9/ml. The percentage of piliated-phase cells was estimated by plating and counting the relative numbers of piliated-phase and nonpiliated-phase colonial types (3, 23). Aliquots of the challenge inocula were stored at −70°C.

Vaccines. Three vaccine preparations were used. The first vaccine contained purified 987P isolated from a piliated-phase variant of strain 987 and was prepared by the procedures of Brinton et al. (4, 11), like the 987P vaccine used previously (24). The second vaccine contained purified K99 (13) prepared from an *E. coli* K-12 strain possessing the K99 plasmid from a bovine ETEC (B41). Purified pili were adjusted to 0.9 mg of protein per ml in saline (0.15 M NaCl) and dialyzed against saline containing 0.05% formaldehyde (11, 24). The third (control) vaccine consisted of saline containing 0.05% formaldehyde.

The physical, chemical, and serological properties of strain 987 pili are described in detail elsewhere (11, 16, 23), as are those of K99 (13). Briefly, 987 pili belong to the general class of somatic pili. They are rodlike assemblies of a single kind of protein subunit with a diameter of 6.9 nm and a subunit molecular weight of 18,900. Although 987 pili are serologically and chemically distinct from the type 1 pilus class of *E. coli* somatic pili, they are purifiable by exactly the same procedure. K99 resembles K88 in that both are plasmid mediated (27, 28). Strain 987 pili resemble type 1 pili morphologically. Type 1 pili are chromosomally mediated (4), and it may be that 987 pili also resemble type 1 pili in this regard. It has been shown (14, 20, 23; C.-C. To et al., manuscript in preparation) that 987P and K99 are serologically unrelated.

Vaccination. Approximately 3 weeks before farrowing, bred gilts were delivered from a source different from that used by Nagy et al. (24). These gilts were assigned to one of three vaccine groups. Each gilt was vaccinated twice subcutaneously in the neck with 10-ml doses of vaccine; the first vaccination was given 16 to 29 days before farrowing, and the second vaccination was given 5 to 17 days before farrowing. No local or systemic reactions were observed after the gilts were vaccinated. Colostrum samples were collected just prior to challenge, clarified by centrifugation at 95,000 × *g*, and stored at −70°C. Antibody titers against O, polysaccharide K, and pilus antigens were determined as previously described (24). O and

K titers were performed by tube agglutination. Pilus antibody titers were determined by agglutination of piliated bacteria, using the microtiter system and plates. Strain 987 was used for 987P titers, and 431 was used for K99 titers (6; C.-C. To et al., manuscript in preparation).

All observations and measurements within each challenge group were performed blind. That is, vaccines and immunized dams were coded to control subjective bias in the clinical and laboratory observations. Colostrum samples were coded before the agglutination titers were determined.

Experimental infections and observations. After birth, the pigs were allowed to suckle (30 min for pigs to be challenged with strain 74-5208 or 6 to 18 h for pigs to be challenged with strains 987 or 431) before challenge. Pigs were challenged with strain 74-5208 at 30 min after birth because in preliminary experiments (when all were exposed at 6 to 18 h after birth) the mortality rate of pigs exposed to strain 74-5208 tended to be lower than that for pigs exposed to strains 987 or 431. Immediately before challenge, each pig was weighed, and any pig weighing less than 800 g was eliminated from the experiment. Up to 12 pigs per litter (pigs in excess of 12 were eliminated) were each challenged intragastrically with 20 ml of fresh TSB containing 2 ml of the appropriate challenge inoculum. All pigs in a litter received the same strain. Challenged pigs were then returned to their dams. Information concerning diarrhea and death was recorded daily. The weight of each pig was recorded on the day of death or day 5 postchallenge if they survived. In addition to these data, on the first day (16 to 29 h postchallenge) the most severely affected pig in each litter (i.e., the pig that lost the most weight or, when no pig lost weight, the pig that gained the least weight) was sacrificed, and a 10-cm portion of the ileum was removed 10 to 20 cm from the ileocecal junction to determine the number of viable challenge organisms at this site. A 1-cm portion of ileum adjacent to the above portion was also removed, embedded in methylcellulose, and used to determine the degree of bacterial adhesion to ileal mucosa by a fluorescent antibody staining technique (22). Adhesion (association index) was graded on a 1-to-5 scale, depending upon the intensity and location of staining; 1 indicated no adhesion, and 5 indicated maximal adhesion. Pigs that died during the experiment were necropsied, and if gross lesions were consistent with enterotoxigenic colibacillosis (21) the pig was considered to have died from that disease. Twelve pigs died with gross lesions not consistent with enterotoxigenic colibacillosis and were excluded from the experimental data, including four deaths from penetrating stomach ulcers, four from starvation, three from trauma, and one from drowning.

RESULTS

The term "homologous vaccine" will be used to indicate the vaccine that contains the same pilus type as the one possessed by the challenge organism, and "heterologous vaccine" will refer to the pilus vaccine that contains a different pilus type from that possessed by the challenge organism. Saline plus formaldehyde vaccine will hereafter be referred to as "control."

***E. coli* 987 (987P$^+$) challenge.** The data pertaining to intestinal colonization (number of viable challenge bacteria in the ileum, association index, and piliation), death, and weight gain for pigs from all three vaccine groups challenged with *E. coli* 987 are summarized in Table 1.

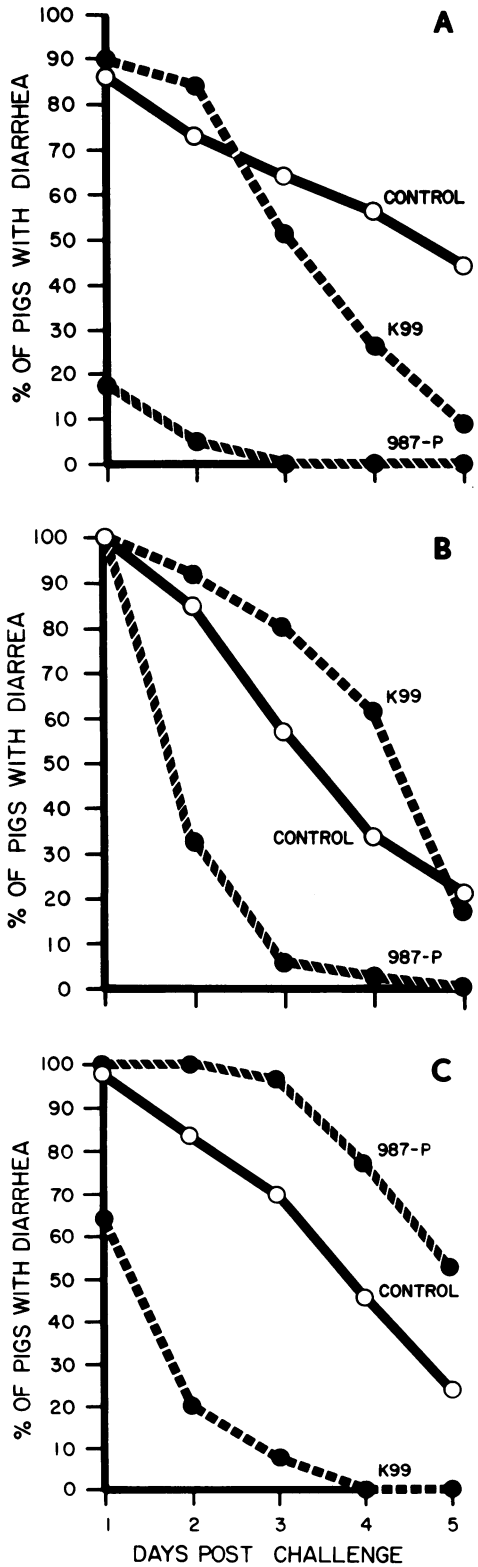
None of the 38 pigs in the homologous vaccine (987P) group that were challenged with strain 987 died. In contrast, 17% of the pigs in the heterologous vaccine (K99) group died, and 27% of the pigs in the control group died. The prevalence of diarrhea among pigs from the three vaccine groups is shown in Fig. 1A. Throughout the 5-day observation period, fewer pigs in the homologous vaccine group had diarrhea than in the other two groups. By day 3, none of the pigs in the homologous vaccine group had diarrhea, while 51% of the surviving pigs in the heterologous vaccine group and 64% of the surviving pigs in the control group still had diarrhea. The prolonged diarrhea in the heterologous and control groups was also reflected by decreased weight gains of surviving pigs (Table 1).

Of the strain 987-challenged pigs sacrificed on day 1, there were statistically fewer viable strain

TABLE 1. *Response of pigs from the three vaccine groups to challenge with E. coli 987*

Vaccine (no. of litters)	1 day postchallenge					5 days postchallenge	
	Challenge *E. coli*/10 cm of ileum			Association index		Death/ total	Weight gain of survivors (%)[a]
	Geometric mean	Range	Piliated-phase variants (%)	Arithmetic mean	Range		
987P (6)	5.4×10^7	2.1×10^7–1.9×10^8	4	1.18	1.1–1.3	0/38	58
K99 (6)	8.4×10^9	6.3×10^9–2.4×10^{10}	91	4.66	4.4–5.0	7/41	39
Control (6)	1.9×10^9	3×10^6–1.4×10^{10}	64	4.22	1.0–4.9	12/44	24
t test[b]	<0.01		<0.0001	<0.0001		<0.01	<0.0001

[a] Percent increase in birth weight.
[b] 987P versus K99 plus control group.



987 bacteria in the ilea of pigs in the homologous vaccine group than in the heterologous vaccine or control groups (Table 1). Most of the colonies from the viable strain 987 bacteria recovered contained cells that were in the piliated phase when isolated from the ilea of pigs in the heterologous vaccine or control groups, whereas most were in the nonpiliated phase when isolated from the ilea of pigs in the homologous vaccine group. The association indexes, which indicate the degree of adhesion of the challenge strain to ileal mucosa, were statistically lower in sections from pigs in the homologous vaccine group than in sections from pigs in the heterologous vaccine and control groups, indicating poor adhesion in the homologous group.

***E. coli* 74-5208 (987P$^+$) challenge.** Data pertaining to pigs from all three vaccine groups challenged with *E. coli* strain 74-5208 are summarized in Table 2 and Fig. 1B. Pigs in the homologous vaccine group (987P) were protected against diarrheal disease, as demonstrated by decreased incidence of death, diarrhea (incidence and duration), and intestinal colonization and increased weight gain, compared to pigs in the heterologous vaccine or control groups. However, 1 of 37 pigs in the homologous vaccine group died of colibacillosis after challenge with strain 74-5208. In contrast to the inocula, which contained 1% piliated-phase bacteria, most colonies of strain 74-5208 recovered from the intestine of pigs in the control and heterologous vaccine groups consisted of bacteria in the piliated phase (Table 2). A similar shift towards the piliated phase after growth in pig intestine was reported for strain 987 (23). Most colonies recovered from pigs in the homologous vaccine group were in the nonpiliated phase.

***E. coli* 431 (K99$^+$) challenge.** Data pertaining to pigs from all three vaccine groups challenged with *E. coli* 431 are summarized in Table 3 and Fig. 1C. Pigs in the homologous vaccine group (K99) were protected against diarrheal disease, as demonstrated by decreased incidence of death and diarrhea (incidence and duration) and increased weight gain, compared to pigs in the heterologous vaccine or control groups. Statistically fewer viable strain 431 bacteria were in the ilea of pigs in the homologous vaccine group than in the heterologous vaccine or control groups. However, all pigs sampled in all three vaccine groups were by our operational defini-

FIG. 1. *Prevalence of diarrhea among pigs challenged with E. coli 987 (A), 74-5208 (B), or 431 (C). Each challenge group contained pig suckling gilts vaccinated with purified 987P pili, purified K99 pili, or control material. Each point represents the percentage of surviving pigs with diarrhea in an entire vaccine group on the day of measurement.*

TABLE 2. *Response of pigs from the three vaccine groups to challenge with E. coli 74-5208*

Vaccine (no. of litters)	1 day postchallenge					5 days postchallenge	
	Challenge *E. coli*/10 cm of ileum			Association index			
	Geometric mean	Range	Piliated-phase variants (%)	Arithmetic mean	Range	Death/total	Weight gain of survivors (%)[a]
987P (5)	7.6×10^7	1.4×10^7–7×10^8	1	1.46	1.0–2.2	1/37	37
K99 (4)	7.6×10^9	3.2×10^9–3.1×10^{10}	56	3.92	3.7–4.5	7/39	10
Control (4)	1.1×10^9	1.2×10^7–5×10^9	90	2.80	1.0–4.2	5/34	23
t test[b]	<0.01		<0.001	<0.01		<0.05	<0.001

[a] Percent increase in birth weight.
[b] 987P versus K99 plus control group.

TABLE 3. *Response of pigs from the three vaccine groups to challenge with E. coli 431*

Vaccine (no. of litters)	1 day postchallenge				5 days postchallenge	
	Challenge *E. coli*/10 cm of ileum		Association index			
	Geometric mean	Range	Arithmetic mean	Range	Death/total	Weight gain of survivors (%)[a]
987P (6)	5.4×10^9	1.7×10^9–1×10^{10}	4.58	4.2–4.9	9/30	−4
K99 (5)	2.4×10^9	1.6×10^9–2.5×10^9	4.26	3.0–4.9	0/41	37
Control (5)	6.7×10^9	6×10^9–1×10^{10}	4.31	3.7–4.7	14/35	9
t test[b]	<0.01		NSS[c]		<0.01	0.0001

[a] Percent increase in birth weight.
[b] K99 versus 987P plus control groups.
[c] NSS, Not statistically significant.

tion considered to be colonized (i.e., $>10^8$ challenge organisms per 10 cm of intestine). Furthermore, the degree of adhesion of strain 431 to ileal mucosa from pigs from all three vaccine groups was indistinguishable. Therefore, even though the homologous vaccine, K99, protected against death and diarrhea after challenge with strain 431, it did not prevent colonization of the terminal ileum by this strain 1 day after challenge. Thus, the following experiment was performed. Pigs from two K99 vaccine litters and one 987P vaccine litter were challenged with strain 431 as described. On a daily basis for 5 days, one pig per litter was sacrificed (the pig selected was the most severely affected on the basis of weight change). Five 10-cm portions of small intestine were removed at equal intervals starting at the usual ileal site and moving cranially until 20 cm posterior to the ligament of Treitz. These segments were evaluated for viable strain 431 bacteria and adhesion. Pigs in the homologous vaccine group were colonized for fewer days than pigs in the heterologous vaccine group (~2 days compared to ~5 days), and colonization was less extensive (only the posterior two segments of pigs in the homologous vaccine group were colonized on day 1, whereas the posterior three segments in the heterologous vaccine group were colonized on day 1).

Antibodies in colostrum. Colostrum from vaccinated gilts had high antibody titers against the pilus type in the vaccine and low antibody titers to the heterologous pili (Table 4). Low antibody titers against both pili were present in the colostrum of the controls. Titers against the O20 and O101 antigens were not significantly different among vaccine groups. In the strain 987 challenge group, the colostrum from gilts vaccinated with 987P had a 3.6-fold higher geometric mean antibody titer to O9 than that for colostrum from the other two groups combined. Statistically, this difference was marginally significant ($P = 0.071$, *t* test; 95% confidence interval for the ratio is 0.84 to 26.3). Antibody titers against polysaccharide K antigens on the challenge organisms were not detected (even in undiluted colostrum).

DISCUSSION

The data indicate that vaccination of pregnant gilts with purified pili provides passive protection of their suckling pigs against diarrheal disease caused by ETEC strains possessing the homologous pilus type but not by ETEC strains possessing heterologous pili. Challenged pigs born to gilts that had been vaccinated with the

TABLE 4. *Agglutinin titers in colostrum against antigens on the E. coli challenge strains*

Vaccine	Test antigen[a]				
	987P	K99	O9	O20	O101
987P	2,314 (17)	19 (17)	453 (6)	24 (5)	40 (6)
K99	<9 (16)[b]	3,444 (16)	<92 (5)[b]	40 (5)	61 (5)
Control	<9 (15)[b]	14 (15)	160 (5)	24 (4)	70 (5)

[a] In each challenge group, colostral titers against O antigen were determined only for the O antigen possessed by the challenge organism. Parentheses indicate number of animals tested.

[b] Geometric mean of the agglutinating titer. To permit calculation of geometric means, the lowest serum dilutions tested were used as the titers for samples that did not cause agglutination. Means which include such samples are preceded by <.

homologous pilus vaccine had lower incidences of death, equal or lower incidences of diarrhea, diarrhea for a shorter duration, and greater weight gain. Furthermore, pigs in the 987P vaccine group, challenged with the two homologous strains, 987 and 74-5208, did not become colonized by these strains. On the other hand, pigs in the K99 vaccine group, when challenged with the homologous strain 431, were colonized; however, the intensity, duration, and extent of intestinal colonization was less than in the other two vaccine groups. Pigs in the heterologous vaccine challenge groups and control groups were not protected from diarrheal disease. In two instances (Tables 1 and 3), survival tended to be higher in the heterologous vaccine group than in the control group. We don't think this was due to protection as the result of heterologous vaccination because in the third instance (Table 2), survival tended to be higher in controls than in the heterologous vaccine group. Furthermore, there were no consistent differences between heterologous and control groups in the incidence and duration of diarrhea, bacterial numbers, or weight gain.

All the mechanisms whereby protection occurs cannot be readily ascertained from the data presented. However, since the vaccinating agents used were highly purified pili, insofar as we could determine (11, 13), it is assumed that immunity arises in pigs from the acquisition of antipilus immunoglobulins via ingested colostrum. The uniform correlation of protection with high antibody titers against homologous pili (and not with those against heterologous pili) is consistent with this assumption. Once in the small intestine, these antibodies could act in a variety of ways. A mechanism that we favor and that is consistent with one hypothesized function of pili is that the antibodies prevent bacterial colonization by inhibiting or reversing bacterial adhesion, or both. The antibodies may also act by agglutinating or opsonizing the challenge bacteria, or by inhibiting other pilus-related functions such as twitching motility and oxygen utilization, or by changing the mode of colonial growth (6, 7). These mechanisms should not be considered as mutually exclusive. The antibody neutralization of more than one pilus function may have enhanced the protection obtained by pilus vaccination. The possibility that the purified pilus preparations may have contained some undetected immunizing antigen(s) that accounts for the protection is recognized. However, this possibility is unlikely because vaccination with either pilus preparation protected against challenge with strains that (except for pili) were (insofar as we could determine) antigenically unrelated to the strains used for vaccine production. We cannot exclude the possibility that protection against strain 987 in the homologous vaccine group was due to the slightly higher O9 antibody titers in colostrum of this group (Table 4). However, this seems unlikely for several reasons. (i) The statistical significance of this difference in O9 titers was marginal ($P = 0.071$; ratio of the difference, 3.6, was within the 95% confidence interval 0.84 to 26.3). (ii) In a previous experiment, this vaccine protected against strain 987 when there were no differences in colostral O9 titers between homologous and control gilts (24). (iii) The 987P vaccine group was also protected against challenge with strain 74-5208 (Table 2), which lacks O9 antigen, and the colostral O20 titers of this protected group were not higher than those of the K99 and control groups (Table 4).

With the reports about vaccinating gilts with K88 (18) and with 987P (24) (confirmed here), our data on K99 vaccination of gilts are a third example of protecting pigs from enterotoxigenic colibacillosis by vaccination of their dams with pili. Since many ETEC strains that cause enterotoxigenic colibacillosis in neonatal pigs apparently possess either K88, K99, or 987P, it is reasonable to hypothesize that vaccinations with pili would reduce the incidence and severity of enterotoxigenic colibacillosis in suckling pigs.

With the reports of Brinton et al. on vaccinating human subjects with a gonococcal pilus vaccine (7) and on vaccinating burned mice with a *P. aeruginosa* pilus vaccine (6, 7), the number of effective vaccines consisting of purified and/or concentrated pili or pilus-like antigens is now five. It is apparent that the direct approach to disease control using pilus vaccines (6) may be a fruitful one for a large number of bacterial diseases.

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