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Change in Degree of Type 1 Piliation of *Escherichia coli* during Experimental Peritonitis in the Mouse

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To determine whether expression of type 1 pili varies during the course of *Escherichia coli* infection in vivo, mice were injected intraperitoneally with 5×10^7 CFU of piliated or nonpiliated phase variants per ml, and the degree of piliation was measured in peritoneal exudate by an enzyme-linked immunosorbent assay inhibition method. In the animals challenged with the piliated bacteria, the numbers of organisms increased a log over 9 h and the amount of pilus antigen decreased from 3 to 0.075 μg/10 bacteria. After a 4-h delay, nonpiliated bacteria also increased by one log over 9 h; however, the amount of piliation remained virtually undetectable. Piliated *E. coli* were more virulent than nonpiliated variants in this model (50% lethal dose of 7.5×10^6 versus 3×10^7, respectively). The difference was significantly reduced by prior passive immunization with rabbit serum containing high titers of antipili antibody. Piliated bacteria adhered in significantly greater numbers to isolated mouse peritoneal membranes than did nonpiliated variants (15,400 ± 2,700 versus 1,300 ± 700 bacteria/mm², respectively; $P = 0.05$). Adherence was inhibited by the presence of 0.1 M alpha methyl mannose (1,500 ± 1,800 bacteria/mm², $P = 0.01$). These results confirm the results of previous qualitative studies showing that phase variation of type 1 pili occurs in vivo and suggest that these pili may confer an initial advantage for growth of *E. coli* in the peritoneal cavity, presumably by fostering colonization of the peritoneal serosal surface.

Pili of gram-negative bacteria are involved in the process of bacterial adherence, an important first step in many infections initiated on mucosal surfaces (2). Type 1, or mannose-specific (MS) pili of *Escherichia coli* have been implicated in the pathogenesis of urinary tract infection. Although their exact role in human disease is the subject of considerable speculation, studies with animal models suggest that they function to permit bacteria to colonize the lower urinary tract (1, 8, 9). Expression of type 1 pili may also render organisms more susceptible to host defense mechanisms such as intravascular clearance (11) and phagocytosis (21), so that pili may either adversely or favorably affect bacterial virulence according to the immediate host environment. In this regard, we have previously shown that piliated *Proteus mirabilis* causes pyelonephritis more readily than do nonpiliated variants when the bacteria reach the kidney by the ascending route (19), whereas the reverse is true when bacteria are delivered directly to the kidney via the hematogenous route (22). Presumably, pili are important for transmucosal infection but are a disadvantage once the bacteria reach the parenchyma and are susceptible to phagocytosis.

Eisenstein has shown that expression of type 1 pili is subject to phase variation controlled by a transcriptional switch which alternates randomly every 10^3 cell divisions (4). The predominant phase of piliation is determined by whether the conditions of growth favor selection of piliated or nonpiliated organisms. In vitro, cultivation in static broth generally produces heavily piliated inocula, whereas growth on solid agar produces nonpiliated colonies (15). From the foregoing considerations, it is clear that the ability to alter the pili phase in vivo would also be of considerable advantage to virulent bacteria encountering different environmental conditions within the host.

Attempts to examine the state of piliation in vivo have been hampered by the lack of sensitivity of methods for quantitating piliated bacteria directly in infected material and by the uncertainty of whether primary culture had altered the expression of pili. In the present study, we used an enzyme-linked immunosorbent assay (ELISA) inhibition technique to measure the change in degree of piliation that occurred during the course of experimental *E. coli* peritonitis. Sufficient concentrations of piliated bacteria were present in the exudate obtained from mice with induced peritonitis to be measured without the need of primary cultivation of the bacteria. The use of this model also provided an opportunity to evaluate the potential role of type 1 pili in the pathogenesis of experimental *E. coli* peritonitis. Our results indicate that type 1 pili potentiate virulence in this infection, apparently by facilitating colonization of the peritoneal surface. Although the numbers of bacteria obtained from the peritoneal exudate increased during the course of the infection, the degree of piliation declined, supporting the concept that significant phase variation occurs in vivo.

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MATERIALS AND METHODS

Bacteria. A clinical urinary isolate of *E. coli* strain 346 (O25:K⁻:H1) was used for all experiments except for the studies of in vitro adherence to the peritoneum, which were performed with another urinary isolate, *E. coli* 2320. Bacteria were quantitated by the pour plate method. Bacterial inocula, containing either predominantly piliated or nonpiliated variants, were obtained by varying the conditions of growth (26). Piliated bacteria were obtained by overnight growth at 37°C in tryptic soy broth (BBL Micro-

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biology Systems, Cockeysville, Md.). After several passages, a rich pellicle was noted on the surface of the broth, indicating heavy piliation. Nonpiliated bacteria were obtained by serial overnight passage at 37°C on Trypticase soy agar plates (BBL). More than 90% of the cells in broth cultures had more than 50 pili when examined by negative-staining electron microscopy, compared with less than 5% of the plate-grown bacteria. Mixture of broth-grown bacteria with a suspension of *Candida albicans* caused the rapid appearance of heavy clumps of the yeast, which was inhibited by the presence of 0.1 M alpha methyl mannoside, indicating that the pili were MS (13).

Purification of pili. Pili were purified by a modification of the method of Brinton et al. (3) as previously described (6). In brief, bacteria were grown in 4-liter flasks in tryptic soy broth for 48 h at 37°C, collected, and washed once in 0.5% saline by centrifugation. Bacteria were resuspended in 0.05 M Tris buffer, pH 8.0, containing 0.2% sodium azide and 30 nM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.). Pili were removed by mechanical shearing in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) at top speed for 10 min on ice. The suspension was centrifuged at 7,000 × *g* for 10 min to remove bacteria, and the supernatant was collected and recentrifuged under the same conditions until no pellet was visible. The supernatant was centrifuged at 15,000 × *g* twice to remove flagella and cell wall fragments. Magnesium chloride was added to a final concentration of 0.1 M, and the solution was left at 4°C for 1 h. The white precipitate of aggregated pili was collected by centrifugation at 12,000 × *g* for 10 min, resuspended in Tris buffer, and centrifuged at 25,000 × *g* for 30 min. The pellet, which contained flagella and cell wall fragments, was discarded, and magnesium chloride was added as described above. These steps of precipitation and differential centrifugation were repeated at least three times or until no pellet of contaminants was observed. The final precipitate was resuspended in 2 ml of Tris buffer. About 30 mg of pili were obtained from 8 liters of broth. The purity of the pili was assessed by the following criteria: only pili crystals were seen on dark-field examination, a single band of characteristic location was found after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and no contaminants were seen upon electron microscopic negative staining.

Production of antisera. Polyclonal antibodies to the pili were raised in rabbits as previously described (20). Rabbits were injected intradermally with 200 μg of purified pili emulsified with an equal amount of Freund complete adjuvant. At 6 to 7 weeks after the first injection, the animals were boosted with a similar dose. When adequate agglutination titers (1:2,000 to 1:8,000 by slide agglutination of piliated bacteria) were achieved, the animals were bled, and the serum was stored at −90°C.

ELISA inhibition techniques. The ELISA inhibition technique was similar to that described by Eisenstein et al. (5). All experiments were performed in phosphate-buffered saline with 0.1% Tween 20 (20-polyoxyethylenesorbitan monolaurate; Sigma Chemical Co., St. Louis, Mo.). All centrifugations were performed in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N.Y.) in 1.5-ml microtest polypropylene tubes (Brinkmann). Pellets were resuspended on a Vortex Genie mixer (American Scientific Products, McGaw Park, Ill.). Antiserum was incubated with the unknown antigen for 60 min at 4°C. Bacteria were washed in phosphate-buffered saline–0.1% Tween 20 by centrifugation three times and incubated with 0.1 ml of a 1:100 dilution of goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Miles-Yeda Ltd., Rehovot, Israel) for 1 h at 4°C. The cells were washed by repeated centrifugation, and 1 ml of solution containing 30 ppm of hydrogen peroxide and 0.05% *O*-phenylenediamine (Sigma) in 5% methanol-water (vol/vol) was added to the bacteria. This preparation was incubated at room temperature in the dark for 1 h. The reaction was stopped with 0.1 ml of 12 N HCl, the tubes were centrifuged, and the optical density of 1 ml of the supernatant was read at a wavelength of 490 nm on a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). Results were expressed as the reduction of optical density from the control tube containing no antigen. To express the degree of piliation as the quantity of pilus protein, a standard curve was constructed which plotted the ELISA inhibition titers of increasing amounts of purified pilus protein. The method detected as little as 100 ng of pilus protein.

Mouse peritonitis model. Animals were infected according to the method described by Smith et al. (24). Female outbred Swiss Webster mice (52) were injected intraperitoneally (i.p.) with 0.5 ml of bacterial suspension containing 25 mg of crude type II porcine stomach mucin per ml (Sigma) and various concentrations of piliated or nonpiliated organisms. Each group comprised at least three mice. The percent mortality at 24 h was observed, and the 50% lethal dose was calculated by the method of Reed and Meunch (17). To determine the effect of antipili antibody on the virulence of piliated organisms, an additional 52 animals were injected with 0.1 ml of rabbit antipili antiserum and challenged 24 h later with piliated or nonpiliated organisms as described above. Also, 48 animals, given 0.1 ml of 0.9% saline or normal rabbit serum in lieu of antibody, served as controls. To quantitate the rate of growth of bacteria within the peritoneal cavity and the change, if any, in their piliation, a third group of animals were injected with 5×10^7 organisms plus mucin. Four mice received piliated *E. coli*, and five mice received nonpiliated *E. coli*. The mice were sacrificed at intervals by cervical dislocation, and the peritoneal exudate was removed by needle aspiration. A 1-ml sample of exudate was centrifuged in the microfuge, and 0.25 ml of the pellet was used for determination of piliation by ELISA. A 0.01-ml sample of exudate was diluted serially, and the number of bacteria was quantitated by the pour plate method.

To confirm the results of the ELISA experiments by direct visualization, negative-stain electron microscopy was performed on bacteria present in the peritoneal exudate. Four mice were injected i.p. with 7.5×10^6 bacteria and mucin as described above. At 6 h, the animals were sacrificed, and the peritoneal exudate was aspirated and fixed in 10% Formalin at 4°C for 2 h. The bacteria and other particulate material were separated by centrifugation in a microfuge for 15 min, fixed in 10% Formalin for 2 h at 4°C, and washed twice with normal saline. A sample of bacteria from the inoculum used for infection was incubated at 37°C for 6 h and processed in the same way. Copper grids coated with formvar and carbon were floated on drops of either the washed peritoneal exudate sediment or incubated bacteria, washed in distilled water, and negatively stained with 1% uranyl acetate. The grids were photographed in a Philips 201 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.).

Quantitation of adherence in vitro. Mice were sacrificed by cervical dislocation, and their abdominal cavities were exposed. Portions of the mesentery were excised and spread between two closely fitting concentric rings. The inner ring was polystyrene (2 by 14 mm; Millipore Corp., Bedford,

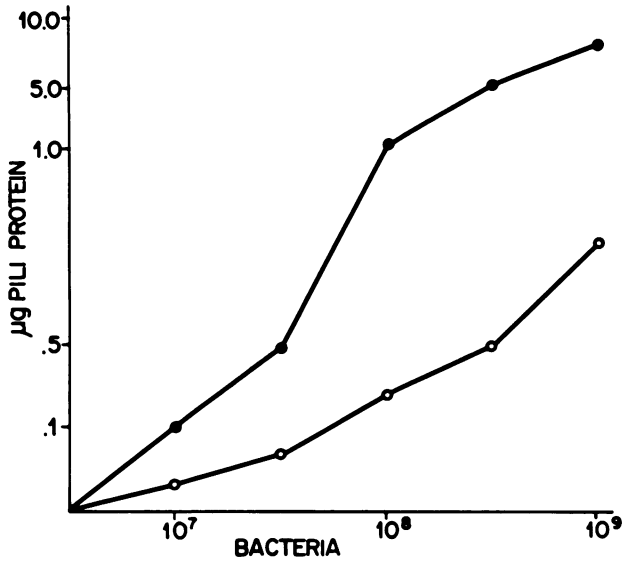

FIG. 1. Amount of pilus protein associated with increasing concentration of broth-grown (○) and plate-grown (●) bacteria.

Mass.), and the outer ring was a rubber faucet O ring (15.6 mm, outer diameter; Kirkhill, Inc., Downey, Calif.). The mesentery and supporting rings were immersed in phosphate-buffered saline containing 10^8 bacteria (piliated or nonpiliated) per ml, with or without 0.1 M alpha methyl mannose at 22°C for 100 min. After incubation, the rings were washed several times in buffer, and the mesenteric membranes were removed and spread on glass slides. The membranes were Gram stained, and the number of adherent bacteria was counted by light microscopy with a calibrated ocular grid. To preserve objectivity, the slides were coded, and the areas of the membrane selected for counting were assigned with the use of a table of random numbers. Differences in numbers of adherent bacteria for different conditions were compared by Student's *t* test.

RESULTS

The amount of pilus protein associated with increasing concentrations of piliated and nonpiliated *E. coli* is displayed in Fig. 1. There was an approximate 10-fold difference in the amount of pili detected in piliated and nonpiliated bacterial inocula, i.e., 10^7 broth-grown bacteria contained 0.1 μg of pilus protein, whereas the same amount of pilus protein was found on 10^8 plate-grown bacteria.

The rate of bacterial growth and change in expression of pili of bacteria injected into the peritoneal cavity of mice is depicted in Fig. 2. At 9 h after inoculation, both broth- and plate-grown bacteria had increased by one log, from 5×10^7 to 5×10^8, although the onset of the rapid proliferation of plate-grown bacteria appeared to be delayed 4 h. Whereas broth-grown bacteria became progressively less piliated during the period of observation, plate-grown bacteria maintained their low degree of piliation. The reduction in degree of piliation of the heavily piliated bacterial inoculum was even more striking when piliation was expressed as a function of the number of bacteria, decreasing in 9 h from 3 to 0.075 μg/10^8 bacteria. Because peritoneal material might have adsorbed to the pili and thereby interfered with detection of pilus antigen by ELISA, we directly examined bacteria in the peritoneal cavity by electron microscopy. The results confirmed those of the ELISA method. Fewer than 15% of the organisms from the peritoneal exudate of animals infected for 6 h had any pili, whereas more than 85% of bacteria incubated in parallel in vitro had many pili (Fig. 3).

Piliated bacteria were more virulent in this model than were nonpiliated organisms. The 50% lethal dose of broth-grown bacteria was 7.5×10^6, whereas for the plate-grown bacteria it was 3×10^7 (Fig. 4). Passive immunization with 0.1 ml of antipili antiserum i.p. 24 h before bacterial challenge significantly reduced this difference in virulence. Normal rabbit serum had no effect.

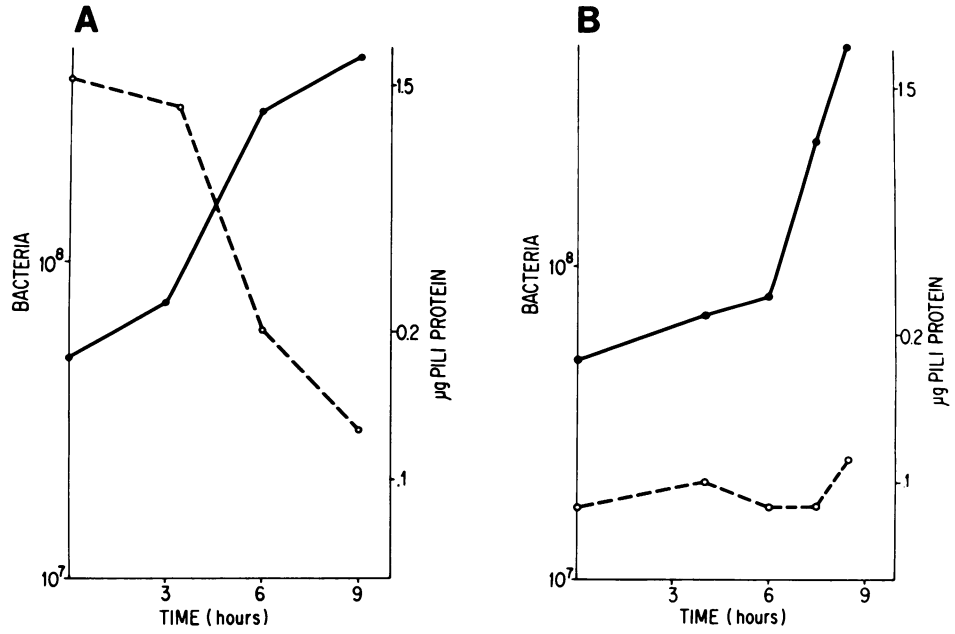

FIG. 2. Change over time in viable counts (——) and degree of piliation (-----) of bacteria in the peritoneal exudates of mice inoculated with 5×10^7 *E. coli*. (A) Animals challenged with broth-grown (piliated) bacteria. (B) Mice inoculated with plate-grown (nonpiliated) organisms.

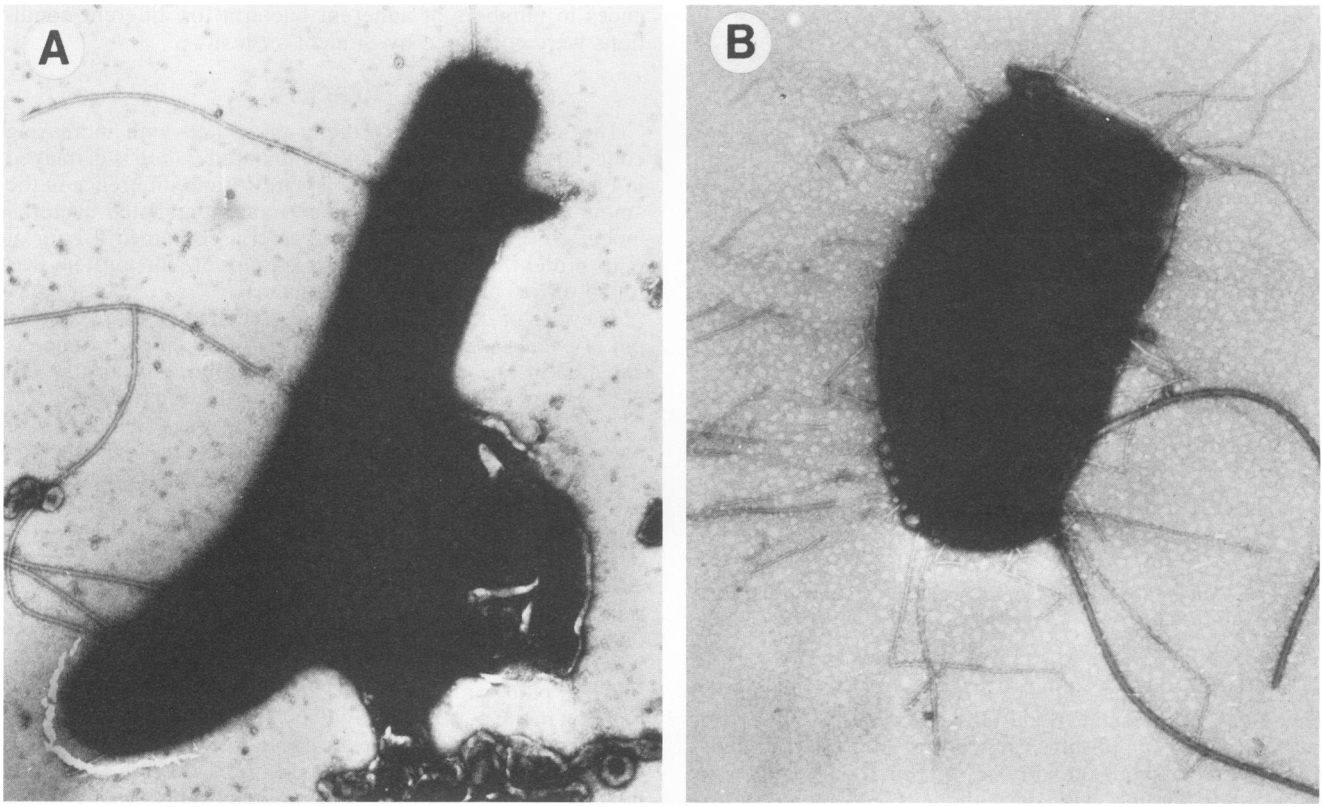

FIG. 3. Electron microscopic negative stain. (A) A nonpiliated bacterium in the peritoneal exudate of a mouse infected 6 h previously with piliated *E. coli*. (B) A piliated bacterium that had been incubated in vitro in parallel with the inocula that were used to infect the mouse. Magnification, ×25,000.

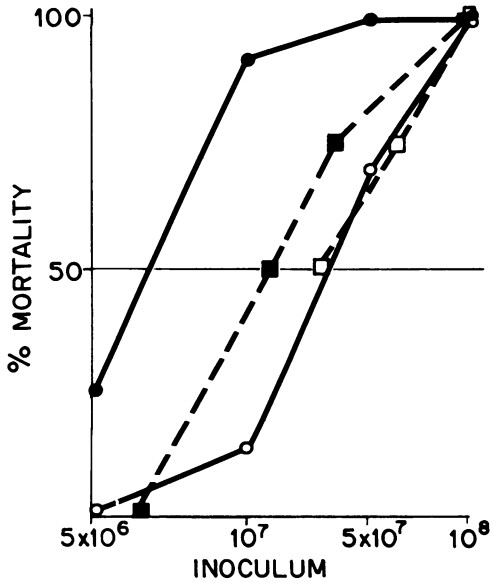

FIG. 4. Lethality of *E. coli* injected into the peritoneal cavity of mice. Symbols: ■ and □, mice which received 0.1 ml of rabbit antipili antiserum i.p. 25 h before infection; ● and ○, unimmunized saline controls. Animals were challenged with broth-grown (filled circles or squares) or plate-grown (open circles or squares) bacteria.

Piliated bacteria adhered in significantly greater numbers to peritoneal mesenteric membranes in vitro than did the nonpiliated variants (Table 1; Fig. 5). Adherence of piliated bacteria was inhibited by 0.1 M alpha methyl mannoside. Adherence appeared to have a detrimental effect on the membranes, causing karyorrhexis and loss of the serosal cell layer in many regions.

DISCUSSION

The major finding of this study was the documentation that *E. coli* can undergo a progressive change in amount of type 1 pili expressed during the course of an experimental infection. Previous investigators have used a variety of predominantly qualitative techniques to investigate whether bacteria in infected tissue were piliated. Stephens et al. used electron microscopy to establish that *Neisseria meningitidis* express pili in the cerebral spinal fluid of patients with meningitis (25). Immunocytochemistry was used by Hultgren et al. to demonstrate that type 1 pili were present on bacteria adher-

TABLE 1. Adherence of piliated and nonpiliated *E. coli* to mouse peritoneal membranes in vitro

Piliation	Inhibitor (alpha methyl mannoside)	Adherence (no. of bacteria/mm²)[a]
MS⁺	−	15,400 ± 2,700
MS⁻	−	1,300 ± 700[b]
MS⁺	+	1,500 ± 1,800[c]

[a] Mean ± standard deviation of at least three membranes.
[b] *P*, 0.005 versus MS⁺ without inhibitor by Student's *t* test.
[c] *P*, 0.01 versus MS⁺ without inhibitor by Student's *t* test.

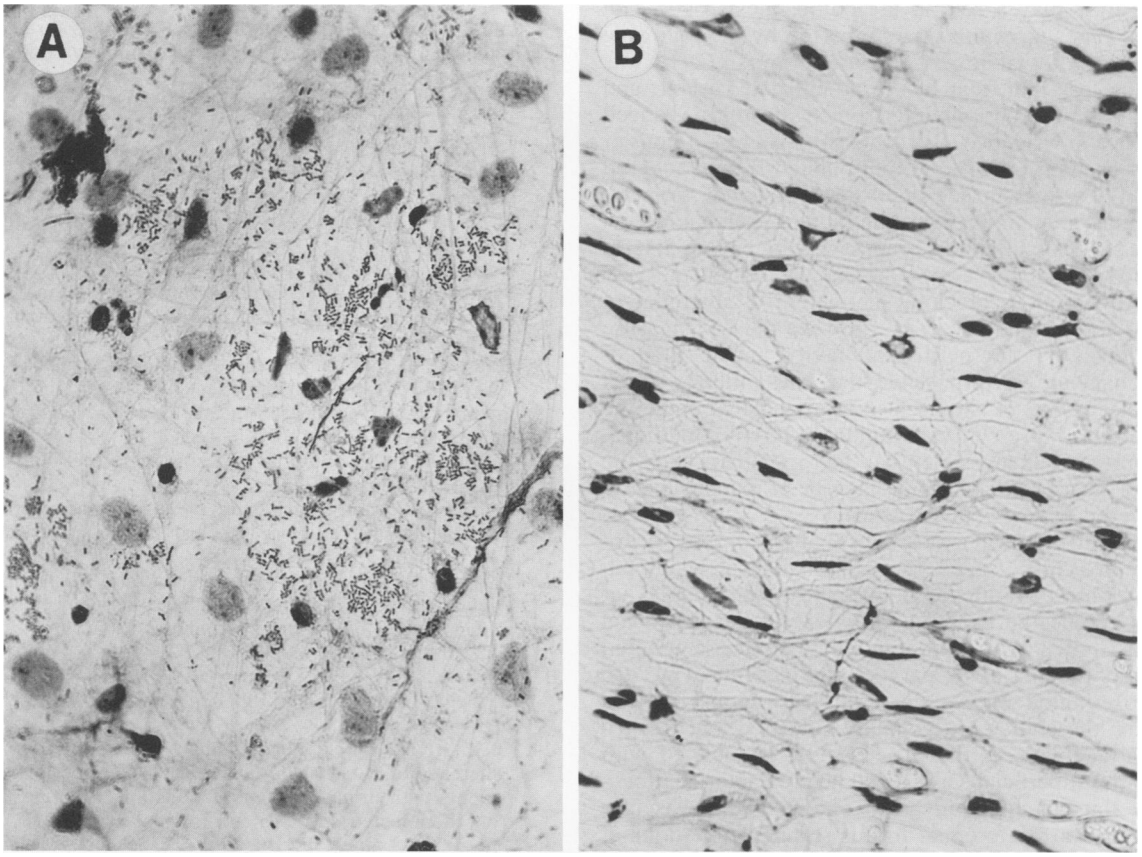

FIG. 5. Peritoneal membranes incubated with piliated (A) and nonpiliated (B) *E. coli*. Note the pale, swollen nuclei of the serosal cells in the membrane exposed to the piliated bacteria (A). Magnification, ×400.

ent to bladder mucosal cells of experimentally infected mice (9). Ofek et al. took advantage of the fact that organisms with type 1 pili agglutinate yeast to show that *E. coli* in freshly voided urine of infected patients were not piliated (14). In the present study, we used an ELISA inhibition method to quantitate the amount of pilus antigen present on *E. coli* infecting the peritoneal cavity of mice. Our method was similar to that of Eisenstein et al. (5). Their technique permits quantitation of the relatively small amount of pilus antigen present in infected material. It can discriminate between individual types of pili present on clinical isolates of *E. coli* and obviates the need for subjecting the sample to manipulations which might alter the degree of piliation. Because it was possible that protein or other substances present in the peritoneal exudate had absorbed onto the pili and thereby interfered with the ability of the pili to interact with the antibody in the ELISA method, we visualized the bacteria directly by negative-stain electron microscopy. Although qualitative, the results confirmed those of the immune assay. Our results confirm those of previous studies which measured piliation on primary isolates. Guerina et al. showed that after oral challenge of neonatal rats, MS^+ *E. coli* predominated in the oral cavity and MS^- *E. coli* were isolated from the bloodstream, no matter what the phase of piliation of the challenging strain (7). Similarly, Maayan et al. found that MS^+ organisms predominated in the urine of mice inoculated intravesicularly with *Klebsiella pneumoniae*, whereas the predominant variant isolated from the kidneys was MS^- (12).

The mechanism responsible for phase variation of type 1 pili of *E. coli* has been shown by Eisenstein (4) to be a genetic switch similar to that described by Silverman and Simon for flagella phase variation in salmonellae (23). The polarity of the switch alternates spontaneously every 10^3 cell divisions, providing a continual source of both piliated and nonpiliated organisms. Environmental conditions determine which phase will overgrow and predominate. In vitro factors, such as whether growth occurs on solid or in liquid media, are believed to be responsible for selective outgrowth of one or the other phase of *E. coli* type 1 pili. For other types of pili, growth at reduced temperature (10) or in the presence of glucose suppresses formation of pili (18).

It is not known what environmental pressures contribute to the selection of piliated or nonpiliated organisms in vivo. One might speculate that at a fluid-surface interface, the two pili phases become partitioned and the piliated bacteria adhere to the surface whereas the nonpiliated bacteria remain in the fluid. The fate of the two populations would then depend on competing selective pressures. At mucosal surfaces, nonadherent bacteria are likely to be removed by the lavaging effect of mucosal secretions, favoring the persistence of piliated bacteria. On the other hand, once bacteria breach the mucosal barrier, or in tissues in which surface adherence plays no role in pathogenesis, the unnecessary metabolic burden of producing pili would likely favor the survival of the nonpiliated phase. Similarly, in the renal parenchyma (22) or the bloodstream (11), MS^+ organisms may be at increased risk of phagocytosis, which could also promote the selection of nonpiliated organisms.

The second outcome of this study was the observation that

type 1 pili may play a role in the pathogenesis of *E. coli* peritonitis. This suggestion was supported by the following findings: the 50% lethal dose of a piliated strain of *E. coli* was almost a log less than that of the nonpiliated variant, and preadministration of antipili antiserum significantly reduced this difference in virulence. By using isolated preparations of intestinal mesentery, we found that piliated *E. coli* adhered more readily to this surface than did nonpiliated variants. The fact that attachment was inhibited by alpha methyl mannoside suggested that this process was mediated, at least in part, by MS ligands. We presume that the antipili antibody afforded protection by blocking pili-mediated adherence at the serosal surface; however, because we did not measure antibody levels in the peritoneal cavity at the time of bacterial challenge, it is possible that the antibody effect occurred elsewhere. It should also be noted that since the two pili variants were cultivated under different conditions, agar or broth, it is possible that factors other than the presence or absence of pili may have been responsible for the observed differences in virulence.

In contrast to the urinary or intestinal tracts, the peritoneal cavity is a closed space; thus, the manner in which pili might contribute to virulence is likely to be different. That type 1 pili enable the bacteria to adhere to the peritoneal surface was shown by the fact that piliated bacteria adhered preferentially to isolated preparations of mesentery in vitro in a mannose-inhibitable manner. Microscopic observations made during the course of this study indicated that in vitro bacterial attachment to the mesentery is associated with damage to the superficial serosal cell layer. Bacterial attachment may thus facilitate invasion of this highly vascular tissue and lead to bacteremia. In this regard, Onderdonk et al. reported that rats experimentally infected i.p. with bowel contents experienced early *E. coli* septicemia, whereas anaerobic abscesses were a late complication in surviving animals (16). Alternatively, the relative delayed onset of rapid growth of the nonpiliated bacterial inoculum suggests that pili may facilitate more rapid growth in vivo, perhaps in a manner analogous to the way biphasic media enhance the recovery of some bacteria from blood cultures. It should be noted, however, that our observations were confined to two strains; thus, the general relevance of our findings to bacterial peritonitis is unknown.

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