JOURNAL OF BACTERIOLOGY, May 1981, p. 784-789
0021-9193/81/050784-06$02.00/0

Vol. 146, No. 2

Escherichia coli 987P Pilus: Purification and Partial Characterization

RICHARD E. ISAACSON* AND PATRICIA RICHTER

Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, Michigan 48109

Received 25 August 1980/Accepted 9 February 1981

The *Escherichia coli* somatic pilus, 987P, has been purified after removal by homogenization from a 987P$^+$ enterotoxigenic *E. coli*. Cell-free pili were precipitated by the addition of $MgCl_2$, collected, and dissolved in $MgCl_2$-free buffer. Five cycles of precipitation and dissolving resulted in a preparation of 987P that was judged to be homogeneous based on electron microscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In the electron microscope, 987P was rod shaped, having a diameter of 7 nm and an apparent axial hole. Cells and membrane vesicles were not observed in the purified pilus preparation. Electrophoresis of 987P through sodium dodecyl sulfate-polyacrylamide gels resulted in a single band when the sample was denatured in the absence of mercaptoethanol and in two bands when the sample was denatured in the presence of mercaptoethanol. The calculated molecular weight of 987 was variable, depending upon the polyacrylamide concentration and whether mercaptoethanol was included in the denaturing solution. Chemically, 987P is composed primarily of protein but also contains an unidentified amino sugar. The amino terminal amino acid of 987P is alanine and its isoelectric point is pH 3.7. 987P possesses no detectable hemagglutinating activity.

One mechanism whereby enterotoxigenic *Escherichia coli* (ETEC) colonize the small intestines of mammals and thus cause severe diarrheal disease is by adhesion to the host gut mucosa. Specific somatic pili have been shown to be responsible for this adhesive property and thus are considered colonization factors (10, 11, 18, 23, 24). One somatic pilus, called 987P (10, 19), has been frequently found on ETEC that adhere to and colonize the small intestines of neonatal pigs (16).

Several lines of evidence support the contention that 987P when present on ETEC causes them to adhere to and thus colonize the mucosa of pig small intestines: (i) diarrheal disease can be experimentally reproduced in neonatal pigs by intragastric challenge with live ETEC strain 987, which produces 987P, but not with nonpiliated mutants of 987 (10, 18); (ii) strain 987 adheres to the mucosa of pig small intestines after intragastric challenge or after introduction into ligated ileal loops, whereas nonpiliated mutants do not (10, 18); (iii) passage through the gut promotes an increase in the number of piliated-phase variants (19); (iv) piliated 987 adhere in vitro to isolated epithelial cells from pig small intestines, whereas nonpiliated mutants do not (9): in vitro adhesion is pilus-specific based upon results from competition experiments using purified pili and by the prevention of adhesion with univalent, pilus-specific antibodies (Fab fragments) (8a). (v) Purified 987P stimulates the production of pilus-specific antibodies in pig colostrum (8a, 17, 20). Consumption of colostrum from pigs vaccinated with 987P by neonatal pigs protects them from severe diarrheal disease when challenged intragastrically with 987P-producing ETEC.

The objectives of this manuscript are to present the procedures used to purify 987P and to present some characteristics of the purified pilus.

MATERIALS AND METHODS

Growth of piliated ETEC strain 987. ETEC strain 987 (O9:K103:NM) is a pig enteropathogen that produces heat-stable enterotoxin and 987P pili. The production of 987P on this organism is subject to pilus phase variation (3, 19). Since the nonpiliated phase is favored by in vitro growth, it is important to inoculate cultures with cells in the piliated phase if production of pili is desired. Strain 987 was streaked onto blood agar plates (Difco blood agar base with 5% sheep blood) incubated at 37°C, and colonies containing cells in the piliated phase were identified by colonial morphology and by agglutination in 987P-specific antiserum (19). Fermentors (Fermentation Specialties) containing 10 to 12 liters of Trypticase soy broth (BBL Microbiology Systems) were inoculated with a single

colony composed of cells in the piliated phase. Growth was for 18 h at 37°C with aeration. No antifoam was used.

Purification of 987P. The procedure used to purify 987P has been briefly described by Brinton (3) for the purification of *E. coli* type I pili. Cells grown as indicated above were collected at 10,000 × *g* at 4°C for 30 min. The pellet was suspended in 100 ml of solubilizing buffer (0.01 M morpholinopropanesulfonic acid [MOPS], pH 7.2), and the pili were removed from the cells by blending for 2 min at 4°C in a Sorvall Omnimixer (setting of 10). Cells were removed by centrifugation at 10,000 × *g* for 30 min at 4°C. The supernatant was adjusted to pH 3.9 by the addition of 1 N acetic acid and stirred for 30 min at room temperature. The resultant precipitate containing pili was collected by centrifugation and resuspended in 100 ml of solubilizing buffer. Insoluble material and any remaining cells were removed by centrifugation. To the clarified supernatant was added an equal volume of crystallizing buffer (0.09 M MOPS, pH 7.2, 0.2 M $MgCl_2$, 1.7% NaCl), which resulted in the precipitation of the pili. After 30 min of stirring, the pili were collected by centrifugation, dissolved in solubilizing buffer, and any insoluble material was removed by centrifugation. The precipitation and solubilization steps were repeated for four additional cycles.

Amino acid analysis. Purified 987P pili were dialyzed exhaustively against distilled water and lyophilized. One milligram of lyophilized material was dissolved in 2 ml of 6 N HCl, placed in an ampule, evacuated and sealed. Individual samples were hydrolyzed for 24, 48, or 72 h at 110°C. After hydrolysis, samples were dried, using a stream of nitrogen gas, and amino acid analysis was performed in a Beckman 121 C automated amino acid analyzer.

***N*-terminal amino acid determination.** The procedure of Gros and Labousse (6) was used to dansylate purified 987P pili. The dansylated protein was hydrolyzed in 6 N HCl in vacu for 4 h at 110°C. The hydrolysate was dried, extracted with water (pH 3.5)-ether (1:1), and both phases dried separately. The residue from each phase was dissolved in pyridine and chromatographed in two dimensions in silica gel-thin layer plates as described by Gros and Labousse (6).

Chemical assays. 2-Keto-3-deoxyoctonate was assayed by the procedure of Osborn et al. (21) and phosphate was assayed by the procedure of Ames and Dubin (1). Neutral sugars were assayed by the indole test (2), and amino sugars were assayed in the amino acid analyzer.

Isoelectric focusing. Isoelectric focusing was performed as previously described (8) with 3% carrier ampholytes (pH 3 to 10).

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-5% polyacrylamide gels were prepared and used according to the method of Weber and Osborn (25). SDS-11% polyacrylamide gels were prepared and used according to the method of Lugtenberg et al. (13). Samples were denatured by the addition of 1 N HCl to pH 2 and boiling for 2 min. The samples were neutralized by the addition of 1 N NaOH, and SDS was added to a final concentration of 2%. 2-Mercaptoethanol, when used, was added to a final concentration of 1%. The samples were then boiled for 5 min. Gels were stained by the method of Fairbanks et al. (5). Bovine serum albumin, pepsin, trypsin, and lysozyme (molecular weights, 68,000, 35,000, 24,000, and 14,600, respectively) were run as protein molecular weight standards.

Electron microscopy. 987P (1 mg/ml) in solubilizing buffer was dropped onto Formvar-carbon-coated grids. After 1 min, excess fluid was removed with filter paper. The grid was floated on distilled water (sample side to the water) for 15 s and then stained for 15 s with 1% sodium phosphotungstate. Excess stain was removed, and the grid was air dried. The sample was observed in a Siemens Elmiskop I electron microscope.

Hemagglutination. Microhemagglutination has been previously described (11). Blood specimens were drawn fresh daily.

RESULTS

Electron microscopy. Figure 1 is an electron micrograph of negatively stained 987P prepared by the procedure described above. Long, slender, rigid filaments characteristic of pili are shown. The pili have diameters of 7 nm each and are of variable length. There is a tendency for the pili to aggregate into parallel bundles. A few pili have what appear to be an axial hole. (These pili are indicated by the arrow in Fig. 1.) Cells and membrane vesicles were not observed in any of the fields that were scanned.

SDS-polyacrylamide gel electrophoresis. Figure 2 shows stained gels of the purified 987P. Tracks A and B are 5% polyacrylamide gels and tracks C and D are 11% polyacrylamide gels. Samples of 987P denatured without the addition of mercaptoethanol (Fig. 2, tracks B and C) migrated as single molecular weight species. The calculated molecular weight for 987P in the 5% polyacrylamide gel (Fig. 2, track B) was 20,000, and in the 11% polyacrylamide gel (Fig. 2, Track C) it was 19,400. No other stained bands were observed. The addition of mercaptoethanol to the denaturing solution resulted in the separation of two 987P components. This effect was most prominent in the 5% polyacrylamide gel (Fig. 1, track A). Two stained bands can be seen; the lower major band consisted of material with a calculated molecular weight of 20,000 and the upper minor band consisted of material with a calculated molecular weight of 26,500. The two-band pattern also was seen in gels of 11% polyacrylamide when higher concentrations of pili were used (data not shown). In other 11% polyacrylamide gels when the minor band was observed (sample treated with mercaptoethanol), the material in it had a calculated molecular weight of 22,000. Another trait characteristic of 987P denatured with mercaptoethanol is shown in Fig. 2, track D. There is an apparent increase in the molecular weight of the 987P protein from

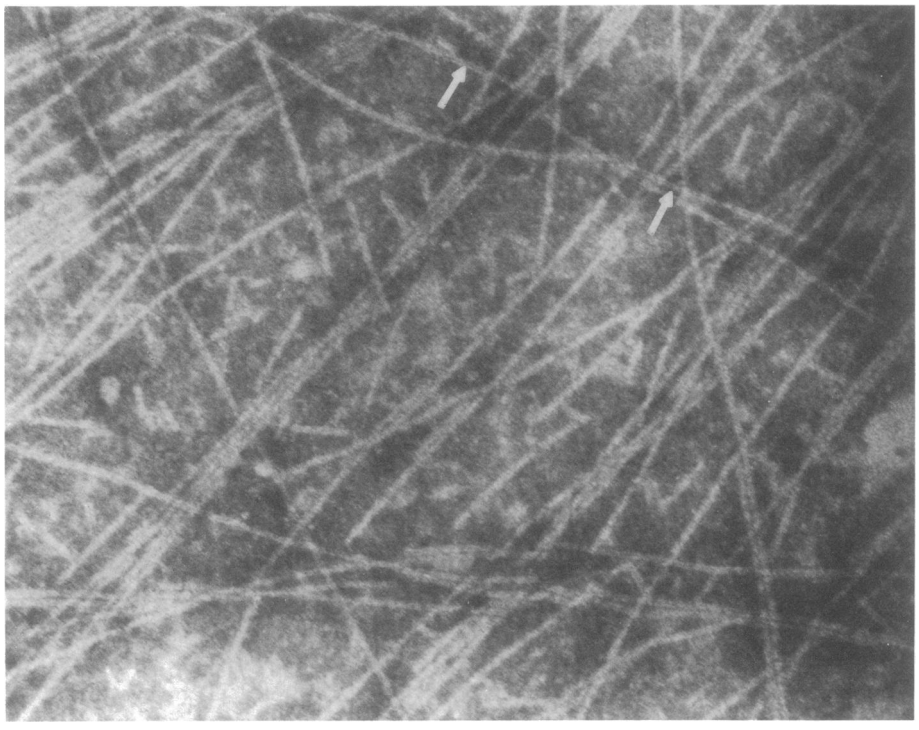

FIG. 1. *Electron micrograph of purified 987P (1 mg/ml) negatively stained as described in the text. Pilus diameters are 7 nm. Arrows indicate pili where axial holes are apparent. Magnification, ×117,000.*

19,400 (without mercaptoethanol) to 20,650 (with mercaptoethanol) (Fig. 2, tracks C and D).

Chemical composition. The amino acid composition of 987P is shown in Table 1. 987P contained 47.6% nonpolar amino acids. There was no detectable histidine, and there were two cysteine residues. The molecular weight of 987P based on amino acid composition is 23,500, which is in close agreement with the data obtained from SDS-polyacrylamide gel electrophoresis.

Samples of 1, 5, and 10 mg 987P were tested for the presence of phosphate, neutral sugars, and 2-keto-3-deoxyoctanate. None of these substances were detected (less then 1.0 μg of phosphate or neutral sugar and less than 0.1 μg of 2-keto-3-deoxyoctonate). However, an unidentified amino sugar was detected in the amino acid analyzer. This amino sugar migrated close to glucosamine.

The amino terminal amino acid of 987P was determined by dansylation of the protein, followed by acid hydrolysis and thin layer chromatography. Dansyl-alanine and dansyl-η-lysine were the only dansylated amino acids detected after thin-layer chromatography.

Isoelectric focusing. Purified 987P was subjected to isoelectric focusing in a pH 3 to 10 gradient (Fig. 3). Material absorbing at 280 nm was found as a major peak with an isoelectric point of pH 3.7. A second smaller peak was found with an isoelectric point of pH 4.5. The peak at pH 4.5 was observed in two of three isoelectric focusing experiments. Whether this peak is an artifact or demonstrates the presence of contaminating proteins is not known. The broad diffuse peak found between pH 1 and pH 3 was attributed to the ampholytes or electrode solution.

Hemagglutination. Purified 987P and piliated ETEC strain 987 were tested for the ability to agglutinate erythrocytes from the following animal sources: horse, guinea pig, rabbit, sheep, pig, or cattle. No agglutination reactions were observed with intact cells, using guinea pig erythrocytes (10) or using the other listed erythrocytes. Erythrocyte agglutinations also were not observed with purified pili. Incubation in the cold (4°C) or at room temperature (22°C) did not promote hemagglutination nor did the addition of D-mannose (0.5%).

DISCUSSION

987P pili have been purified and shown to be primarily protein in composition. No cells or membrane vesicles were seen by electron mi-

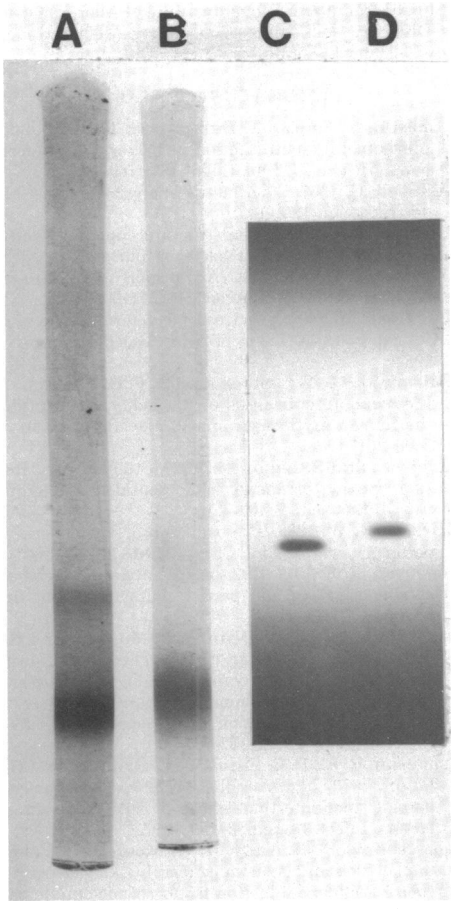

FIG. 2. *SDS-polyacrylamide gels of purified 987P. Tracks A and B are 5% polyacrylamide gels and tracks C and D are 11% polyacrylamide gels. Samples in tracks A and D were denatured with the addition of mercaptoethanol, whereas samples in tracks B and C were denatured without mercaptoethanol.*

croscopy in the purified 987P preparation, and no contaminating proteins were detected by electrophoresis. The only detectable non-amino acid component was an amino sugar. Based upon the results of electron microscopy and SDS-polyacrylamide gel electrophoresis, we believe that the 987P prepared is homogenous. The detection of a single amino terminal amino acid, alanine, is also consistent with the assumption that 987P is homogenous.

Like other pili, 987P appears to be composed of protein subunits, called pilin, that are assembled into the long, rod-shaped structures observed by electron microscopy. The molecular weight of 987P pilin is approximately 20,000, based on the data from SDS-polyacrylamide gel electrophoresis. The molecular weights of pilin from other pili range from 14,500 for CFA/I (12) to 26,200 for K88 (15).

Morphologically, 987P are identical to *E. coli* type I pili. Both are rigid pili with diameters of 7 nm and have what appears to be an axial hole (3). Both pili have the same amino terminal amino acid, alanine (12), and have similar isoelectric points (pH 3.7 for 987P pH 3.9 for type I) (3). Furthermore, both pili are purified by the same procedures. On the other hand, the two

TABLE 1. *Amino acid composition of 987P*[a]

Amino acid	No. of residues
Lysine	10
Arginine	2
Aspartic	34
Threonine[b]	28
Serine[b]	23
Glutamic	16
Proline	8
Glycine	26
Alanine	26
Cysteine[c]	2
Valine	15
Methionine	1
Isoleucine	12
Leucine	17
Tyrosine	5
Phenylalanine	3
Tryptophan[d]	1

[a] Based on one methionine. Numbers are the closest integral numbers obtained by averaging the duplicate values for 24-, 48-, and 72-h hydrolysates, except where noted.

[b] Values obtained by extrapolation to zero time.

[c] Determined as cysteic acid after oxidation by performic acid (7).

[d] Determined spectrophotometrically (4).

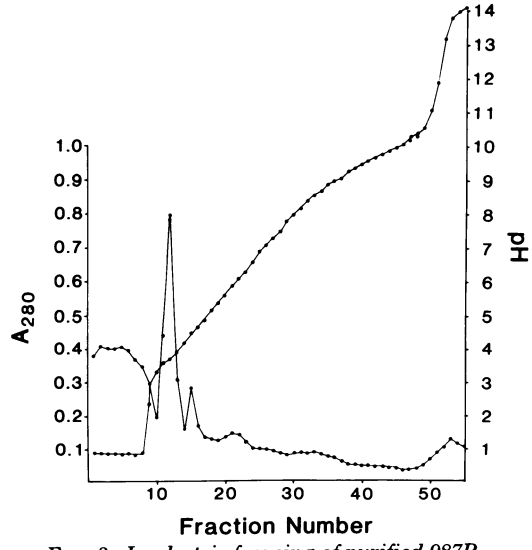

FIG. 3. *Isoelectric focusing of purified 987P.*

pili are antigenically unrelated, are composed of pilin of different molecular weights (20,000 for 987P and 17,100 for type I [3, 14]), and different amino acid compositions (3, 22). Yet, both 987P and type I pili exhibit the same mercaptoethanol effects (14), (i.e., two-band pattern and molecular weight shift). Another *E. coli* pilus, called K99, that is unrelated to 987P and type I pili based on morphology, isoelectric point, pilin molecular weight, amino acid composition, and antigenicity, also exhibits the two described mercaptoethanol effects (8). The three pili, K99, 987P, and type I, each have two cysteine residues, and therefore it is concluded that the observed mercaptoethanol effects are a result of disulfide bond cleavage and reformation. It is presumed that the disulfide bonds in 987P are intrachain. If the disulfide bonds were interchain then it would be expected that, at a minimum, pilin dimers would be produced upon denaturation in the absence of mercaptoethanol. However, in SDS-polyacrylamide gels no bands containing material with a calculated molecular weight of 40,000 or larger were observed. McMichael and Ou (14) concluded that the disulfide bonds in type I pili were intrachain and that the observed mercaptoethanol effects were due to changes in pilin conformation resulting from cleavage and reformation of the disulfide bond or from cleavage only. It should be noted, however, that two other *E. coli* pili, CFA/I and K88, do not contain any cysteine and do not exhibit the mercaptoethanol effects (12, 15; Isaacson, unpublished result). The discrepancy in the molecular weights between the upper minor bands in 5% SDS-polyacrylamide gels (26,500) and 11% SDS-polyacrylamide gels (22,000) is not understood.

The observation that 987P is not a hemagglutinin confirms our previously published observations to that effect (10). Even though the number of species of erythrocytes used was not exhaustive, it did include many types. We cannot rule out the possibility that some species of erythrocytes may be agglutinated by 987P; however, it is also possible that 987P simply is not a hemagglutinin. This raises the question as to the usefulness of using hemagglutination, especially D-mannose-resistant hemagglutination, to identify pili involved in in vivo adhesion. Such procedures, which have been used routinely in searches for these adhesive factors, may be useful as ancillary tools. The true role of hemagglutinins in adhesion to native target cells, however, still requires rigorous proof.

ACKNOWLEDGMENTS

This work was performed with the technical assistance of J. Colmenero.

The work was supported by Public Health Service grant AI 16191-01 from the National Institute of Allergy and Infectious Diseases, U. S.-Japan Cooperative Medical Program, and by Salsbury Laboratories, Charles City, Iowa.

LITERATURE CITED

1. **Ames, B. N., and D. T. Dubin.** 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. **235**:769–775.
2. **Ashwell, G.** 1957. Colorimetric analysis of sugars. Methods Enzymol. **3**:73–105.
3. **Brinton, C. C.** 1965. The structure, function, synthesis, and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram negative bacteria. Trans. N.Y. Acad. Sci. **27**:1003–1054.
4. **Edelhoch, H.** 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. Biochemistry **6**:1948–1954.
5. **Fairbanks, G., T. L. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**: 2606–2617.
6. **Gros, C., and B. Labousse.** 1969. Study of the dansylation reaction of amino acids, peptides, and proteins. Eur. J. Biochem. **7**:463–470.
7. **Hirs, C. H. W.** 1965. The oxidation of ribonuclease with performic acid. J. Biol. Chem. **219**:611–621.
8. **Isaacson, R. E.** 1977. K99 surface antigen of *Escherichia coli*: purification and partial characterization. Infect. Immun. **15**:272–279.

8a. **Isaacson, R. E., E. A. Dean, R. L. Morgan, and H. W. Moon.** 1980. Immunization of suckling pigs against enterotoxigenic *Escherichia coli*-induced diarrheal disease by vaccinating dams with purified K99 or 987P pili: antibody production in response to vaccination. Infect. Immun. **29**:824–826.

9. **Isaacson, R. E., P. C. Fusco, C. C. Brinton, and H. W. Moon.** 1978. *In vitro* adhesion of *Escherichia coli* to porcine small intestinal epithelial cells: pili as adhesive factors. Infect. Immun. **21**:392–397.
10. **Isaacson, R. E., B. Nagy, H. W. Moon.** 1977. Colonization of porcine small intestines by *Escherichia coli*: colonization and adhesion factors of pig enteric pathogens that lack K88. J. Infect. Dis. **135**:531–539.
11. **Jones, G. W., and J. M. Rutter.** 1974. The association of K88 antigen with haemagglutinating activity in porcine strains of *Escherichia coli*. J. Gen. Microbiol. **84**: 135–144.
12. **Klemm, P.** 1979. Fimbrial colonization factor CFA/1 protein from human enteropathogenic *Escherichia coli* strains. FEBS Lett. **108**:107–110.
13. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the 'major outer membrane protein' of *Escherichia coli* K12 into four bands. FEBS Lett. **58**:254–258.
14. **McMichael, J. C., and J. T. Ou.** 1979. Structure of common pili from *Escherichia coli*. J. Bacteriol. **138**: 969–975.
15. **Mooi, F. R., and F. K. de Graaf.** 1979. Isolation and characterization of K88 antigens. FEMS Microbiol. Lett. **5**:17–20.
16. **Moon, H. W., E. M. Kohler, R. A. Schneider, and S. C. Whipp.** 1980. Prevalence of pilus antigens, enterotoxin types, and enteropathogenicity among K88-negative enterotoxigenic *Escherichia coli* from neonatal pigs. Infect. Immun. **27**:222–230.
17. **Morgan, R. L., R. E. Isaacson, H. W. Moon, C. C. Brinton, and C.-C. To.** 1978. 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. Infect. Immun. **22**:771–777.
18. **Nagy, B., H. W. Moon, and R. E. Isaacson.** 1976.

Colonization of porcine small intestine of *Escherichia coli*: ileal colonization and adhesion by pig enteropathogens that lack K88 antigen and by some acapsular mutants. Infect. Immun. **13**:1214–1220.

19. **Nagy, B., H. W. Moon, and R. E. Isaacson.** 1977. 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*. Infect. Immun. **16**:344–352.
20. **Nagy, B., H. W. Moon, R. E. Isaacson, C.-C. To, and C. C. Brinton.** 1978. Immunization of suckling pigs against enteric enterotoxigenic *Escherichia coli* infection by vaccinating dams with purified pili. Infect. Immun. **21**:269–274.
21. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*: isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962–3972.
22. **Salit, A. E., and E. C. Gotschlich.** 1977. Hemagglutination by purified type I *Escherichia coli* pili. J. Exp. Med. **146**:1169–1179.
23. **Smith, H. W., and M. A. Linggood.** 1971. Observations on the pathogenic properties of the K88, HLY, and ENT plasmids of *Escherichia coli* with particular reference to porcine diarrhea. J. Med. Microbiol. **4**:467–485.
24. **Smith, H. W., and M. A. Linggood.** 1972. Further observations on *Escherichia coli* enterotoxins with particular regard to those produced by atypical piglet strains and by calf and lamb strains: the transmissible nature of these enterotoxins and of a K antigen possessed by calf and lamb strains. J. Med. Microbiol. **5**:243–250.
25. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**: 4406–4412.