HEMAGGLUTINATION BY PURIFIED TYPE I *ESCHERICHIA COLI* PILI*

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Agglutination of erythrocytes by *Escherichia coli* (1), Shigella (2), Salmonella (3), and Klebsiella (4) has been known for many years. More recently, the gonococcus has been associated with hemagglutination (HA)[1] (5) and, by using purified pili, it has been conclusively demonstrated that these appendages are the hemagglutinin (6). For coliform bacteria, HA has in some instances been attributed to nonpili components of the bacterial cell (7), but in other studies, using whole organisms, pili have been implicated (2–4). HA presumed to be caused by pili is inhibited by low concentrations of mannose, but that related to nonpili components is unaffected by mannose.

Pilated and nonpilated bacterial variants were used in these earlier HA studies, but this approach does not permit detailed analysis. Purified pili must be used to prove conclusively that mannose-sensitive HA is caused by pili and to investigate further the characteristics of such binding.

An outline of a method for type I pili isolation has previously been reported (8), but there have been no subsequent studies on the adherence of purified type I pili to mammalian cells. We have modified Brinton's isolation procedure and have prepared large yields of type I pili from *E. coli* K12. These pili are pure by electron microscopy, gel electrophoresis, and isopycnic centrifugation, and they agglutinate erythrocytes from several species. HA is inhibited by anti-pili antibodies and saccharides related to D-mannose.

Materials and Methods

Bacteria. A strain of *E. coli* K12 (kindly supplied by Dr. C. C. Brinton, Jr., University of Pittsburg, Pittsburg, Pa.) was used which has no sex pili or flagella. It segregates into two distinct colonial variants as previously described (9): a round glistening colony with clearly defined margins which contains only pilated organisms (P+) and a larger dull colony with ragged edges containing only nonpilated organisms (P−) (Fig. 1).

Colonies of appropriate morphology were selected until agar plates contained 99% P+ or P−. For HA studies, a single colony was selected, spread on nutrient agar plates, and grown for 16–18 h at 41°C to ensure colonial stability. Bacteria were removed from agar plates with sterile swabs,

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[1] *Abbreviations used in this paper*: AMM, α-CH_3-D-mannopyranoside; BSA, bovine serum albumin; CFU, colony-forming units; Con A, concanavalin A; HA, hemagglutination; HAI, hemagglutination inhibition; MHC, minimal hemagglutinating concentration; P+, pilated bacteria; P−, nonpilated bacteria; PBS, 0.01 M sodium phosphate 0.15 M NaCl, pH 7.4; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

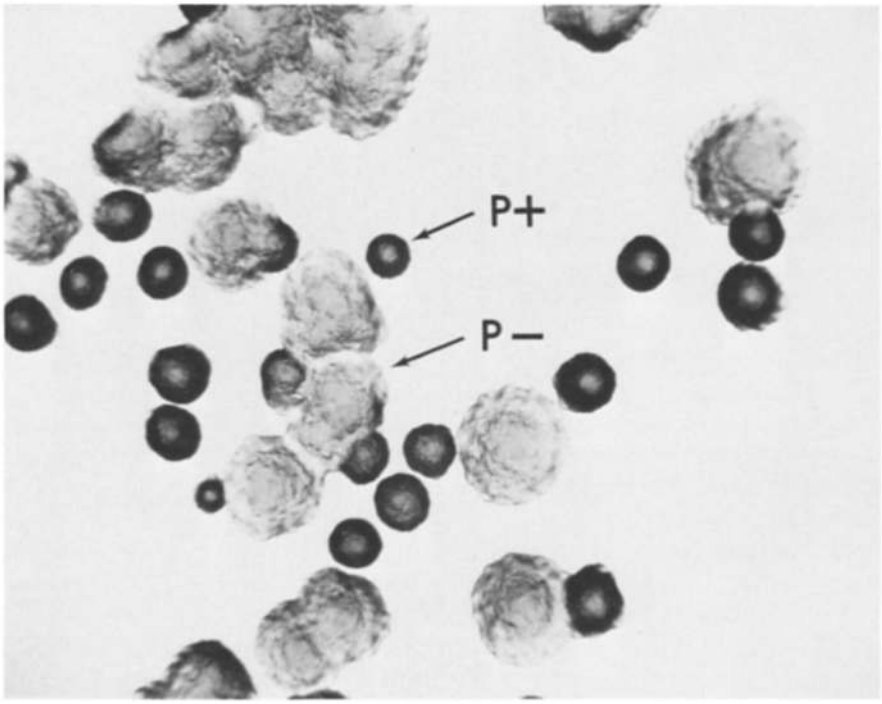

FIG. 1. Pilated (P+) and nonpilated (P−) colonial variants of *E. coli* K12 grown on nutrient agar for 18 h (× 12).

suspended in phosphate-buffered saline, pH 7.4, containing 1% bovine serum albumin (PBS-BSA) and the optical density adjusted to the appropriate value using a Perkin Elmer linear absorbance spectrophotometer (Coleman Instruments Div., Perkin Elmer Corp., Haywood, Ill.). At a wavelength of 540 nm and a tube diameter of 15 mm, an optical density of 0.1 corresponded to a concentration of 1.47×10^8 colony-forming units/ml.

Purification of Type I Pili. The pilated colonial form was selected on nutrient agar and a single P+ colony inoculated into a 2,800 ml Fernbach flask containing 2,000 ml of minimal glucose medium [$MgSO_4 \cdot 7H_2O$, 0.025 g/liter; $(NH_4)_2SO_4$, 1 g/liter; K_2HPO_4, 7 g/liter; sodium citrate $\cdot 2H_2O$, 0.5 g/liter; glucose, 2 g/liter] which was incubated at 41°C at 60 rpm in a gyratory shaker (New Brunswick Scientific, Co., Inc., New Brunswick, N. J.) for 36–48 h. Gram stain, subculture onto nutrient agar and electron microscopy were done on these cultures before final isolation of pili. Alternatively, P+ grown for 16–18 h in 150 × 15 mm Petri dishes containing nutrient agar were used by harvesting colonies with bent sterile glass rods.

P+ grown in liquid culture were collected by centrifugation at 10,000 *g* at 4°C for 20 min in a Sorvall RC 2-B refrigerated centrifuge (Dupont Instruments, Sorvall Operations, Newtown, Conn.) and resuspended in ice cold 0.05 M Tris-HCl, pH 7.8, to a concentration of 75 mg (wet weight) bacteria/100 ml buffer. Bacteria harvested directly from plates were suspended in the same buffer. These bacterial suspensions were then mixed at top speed for 2 min in a Sorvall Omnimixer (Dupont Instruments, Sorvall Operations) with the vessel immersed in ice. The depilated bacteria were centrifuged out at 10,000 *g* at 4°C for 20 min. The supernate was decanted and spun at 10,000 *g* for 30 min to remove any remaining bacteria. This solution was then dialyzed against 0.1 M acetate buffer, pH 3.9, and the aggregated pili removed by centrifugation at 2,000 *g* for 20 min. The sediment was washed in acetate buffer and the pellet resuspended in 0.05 M Tris-HCl, pH 7.8, by stirring rapidly with a magnetic bar at 4°C. Saturated ammonium sulfate was filtered and added dropwise to this stirred solution to a final volume of 10% (at which point strong streaming birefringence is seen). Pili aggregates were then collected by centrifugation at 4,000 *g* for 15 min and resuspended in Tris buffer. Several cycles of such precipitation yield a preparation

containing predominantly type I pili but also containing a major outer membrane protein (subunit size 38,000). The average yield for this procedure is about 35 mg pili from 100 g (wet weight) of bacteria. Further purification was carried out for most subsequent studies by isopycnic centrifugation in cesium chloride: pili were mixed with cesium chloride so that the mixed density was 1.30 g/cm^3 and the pili concentration 100 μg/ml. Centrifugation was then carried out using cellulose nitrate tubes at 4°C with an SW 27.1 swinging bucket rotor at 25,000 rpm for 68 h in a Beckman Model L5-65 ultracentrifuge (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). The purified pili, seen as a dense white band, were collected by puncturing the cellulose tube and monitoring fractions by Folin determinations (10).

Electron Microscopy. Carbon-strengthened, Formvar-coated copper grids (no. 150 mesh) were treated by glow discharge just before use. A drop of a pilus suspension was applied to the surface of an agar-coated slide and a copper grid floated upside down on the specimen drop for several minutes, until most of the fluid was absorbed into the agar. This ensured entrapment of pili in addition to other contaminants which may be present and has previously been used for concentration of immune aggregates (11). Grid preparations were then negatively stained with 2% potassium phosphotungstate, pH 7.0, for 10–15 s. Excess stain was removed by gently blotting one corner of the grid and, after drying, specimens were viewed in a Siemens 101 electron microscope (Siemens Corp., Medical/Industrial Groups, Iselin, N. J.) at 60 kV.

HA. Erythrocytes for agglutination studies were collected from guinea pigs by cardiac puncture and from normal human volunteers by venipuncture. 5–10 ml of blood was mixed with 1 ml of 20 mM EDTA, which was used as anti-coagulant. They were then washed twice in PBS, pH 7.4, and once in PBS-BSA. Cells were suspended to a concentration of 2% for slide agglutination or 0.25% for agglutination in microtiter plates and were used on the day of collection.

To screen for HA, slide tests were done by mixing equal volumes of 2% erythrocytes and the agglutinin (semipurified pili or whole bacteria). To screen for hemagglutination inhibition (HAI), the putative inhibitory substance was preincubated with an equal volume of 2% erythrocytes, and the agglutinin was then added. Agglutination usually occurred within 30–60 s.

To determine HA titers, round bottom 3.5 × 5 inch microtiter plates were used (Linbro "Disposo Trays"; Linbro Chemical Co., New Haven, Conn.). Serial twofold dilutions of the agglutinin were made in PBS-BSA so that 25 μl remained in each well. To each of these wells was then added 25 μl of a 0.25% concentration of the test erythrocytes, the trays were sealed with acetate tape, agitated for 30 s and incubated at 24°C for 2–3 h, at which time end points were recorded. If end points were not clear, the plates were agitated and incubated for an additional 2–3 h. After reagitation and incubation, previously clear end points did not change. If end points still were not clear, the final titer was assumed to be intermediate between the last clear agglutination pattern and the following questionable pattern. Titers were recorded as the arithmetic mean of the minimum concentrations of pili or viable bacteria to yield HA.

HAI titers were determined as follows. To twofold dilutions of the inhibitor (25 μl/well), 25 μl of the test erythrocytes were added, and the plates were sealed and agitated for 30 s. 25 μl of a purified pili solution diluted to contain four times the minimal hemagglutinating concentration (MHC) was then added to each well, and the trays were again sealed, agitated, and agglutination reactions recorded after 2–3 h incubation at 24°C. Inhibitors were compared by recording the arithmetic mean of the minimum molar concentrations that inhibited HA.

The following saccharides were used for inhibition studies: D-mannose, α-CH_3-D-mannopyranoside (AMM), mannitol, α-CH_3-D-glucopyranoside, yeast mannan, and *N*-acetyl-D-mannosamine (Sigma Chemical Co., St. Louis, Mo.); D-lactose, D-fructose, and D-galactose (Fisher Scientific Co., Fairlawn, N. J.); D-glucose (J. T. Baker Chemical Co., Pillipsburg, N. J.); D-fucose, L-fucose, and L-mannose (P-L Biochemicals, Inc., Milwaukee, Wis.); sucrose and maltose (Mallinckrodt Inc., St. Louis, Mo.).

Enzyme Treatment of Erythrocytes. Cells were adjusted to a concentration of 50% in PBS, pH 7.4, containing the appropriate enzyme so that the final concentration was 200 μg/ml of trypsin (EC 3.4.4.4; Worthington Biochemical Corp., Freehold, N. J.) and 1,000 μg/ml for Protease type I (Sigma Chemical Co.). The mixture was incubated at 37°C for 60 min while rotating end over end at 15 rpm.

To treat cells with glycosidases, PBS was used at pH 6.0 and the concentration of cells adjusted to 10%. Final enzyme concentrations were: neuraminidase (EC 3.2.1.18), 0.6 U/ml, β-galactosidase (EC 3.2.1.23) 1 U/ml, β-glucosidase (EC 3.2.1.21), 1 U/ml all obtained from Worthington Biochemi-

cal Corp.; α-mannosidase, 1 U/ml (EC 3.2.1.24; Sigma Chemical Corp.); α-galactosidase, 1 U/ml (EC 3.2.1.22; Boehringer Mannheim Corp., New York), mixed glycosidases, 6.25 mg/ml (Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.). These mixtures were rotated at 37°C for 30 min and an appropriate blank always included. Reactions were stopped by adding 10 volumes of ice-cold PBS. Cells were then washed twice in PBS and suspended to a concentration of 0.25% for use in HA.

Antibodies. For the production of anti-pili antibodies, New Zealand red rabbits were injected intravenously with increasing doses of pure pili (25–300 μg) in PBS every 2–3 days for 10 days, at which time they were bled. Booster doses of 200 μg were given 3–4 days before subsequent bleedings. A similar schedule was followed for injection of formalin-killed P+ and P−. Because these rabbits have natural antibodies to the guinea pig erythrocytes used, all rabbit sera were absorbed with guinea pig erythrocytes before use in HA reactions: guinea pig cells were washed four times in PBS and 2 ml of the packed cells were suspended in 8 ml of heat-inactivated rabbit serum. The mixture was incubated at 24°C for 15 min while rotating at 15 rpm. All erythrocytes were removed by centrifugation at 4,000 *g* for 15 min. The sera were sterile filtered and kept at 4°C.

Immunodiffusion was done on microscope slides covered with 0.6% agarose in PBS. 5-μl samples were added to each well and the slides stored in a moist chamber at 24°C. Precipitin lines were seen at 16–20 h at which time the slides were successively washed in saline and distilled water, air-dried, and then stained with Coomasie Brilliant Blue.

Amino Acid Analysis. 200 μl of 4 N methanesulfonic acid (12) (Pierce Chemical Co., Rockford, Illinois) were added to 2 mg (approximately 120 nmol) of purified pili in a 12 × 75 mm acid-cleaned Pyrex tube. The tube was sealed under vacuum and the pili hydrolyzed for 24 h at 110°C. The tube was then opened and 0.7 ml Durrum buffer, pH 2.2, added, followed by 120 μl of 4 N NaOH. The sample was diluted to 30 nm/ml in Durrum buffer and the analysis carried out in a Jeol amino acid analyzer (Model JLC-6AH; Jeol Ltd., Tokyo, Japan). Cystine was determined as cysteic acid after performic acid oxidation. The quantity of amino acids eluted was determined by comparison with a standard amino acid mixture using a computing integrator (Autolab, Mountain View, Calif.).

Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) was carried out in 12.5% polyacrylamide at a constant current of 25 mA using the method of Laemmli (13). 20-μl samples were boiled for 2 min in a mixture of 10 μl 2% SDS, 10% glycerol, 10 mM Tris-HCl, and 1% 2-mercaptoethanol, pH 7.5. After cooling, 10 μl bromphenol blue in 30% sucrose was added and the sample applied to the gel. Gels were stained with 0.25% Coomasie Brilliant Blue in 50% isopropyl alcohol and 7% acetic acid at 40°C for 1 h. Destaining was done in 20% propanol and 7% acetic acid at 40°C for 3–4 h.

Assays. Concentration of pili was measured by the Lowry method using crystalline BSA as a standard (10), and concentration of saccharides was determined by the phenol sulfuric acid method (14). The activity of glycosidases was measured by release of *p*-nitrophenol from the saccharide, and the thiobarbituric acid assay (15) was used to determine neuraminidase activity. Concentrations of lectins were measured by using $E^{1\%}_{280}$ of 13 for concanavalin A (Con A) (16) (Miles-Yeda, Miles Laboratories, Inc., Elkhart, Ind.) and $E^{1\%}_{280}$ of 12.6 for *Lens culinaris* which was prepared from lentil beans by the method of Howard et al. (17).

Results

Characterization of Isolated Pili. Pili can be aggregated by a variety of methods, including incubation in 0.15 M NaCl, 0.1 M $MgCl_2$, or 10% ammonium sulfate at 4°C, or dialysis against buffer of low pH (less than 4). Individual pili are 7.0 nm in diameter and 1.5 μm long and cannot be seen under the light microscope, but when pili aggregate, they do so side-to-side and these aggregates can be seen as needle-like objects using darkfield (Fig. 2) or phase microscopy. This was used as a convenient method to detect visible impurities (amorphous material) and suitable conditions for aggregation and disaggregation. After several cycles of ammonium sulfate precipitation, amorphous material can still be found in all preparations, but by electron microscopy almost all

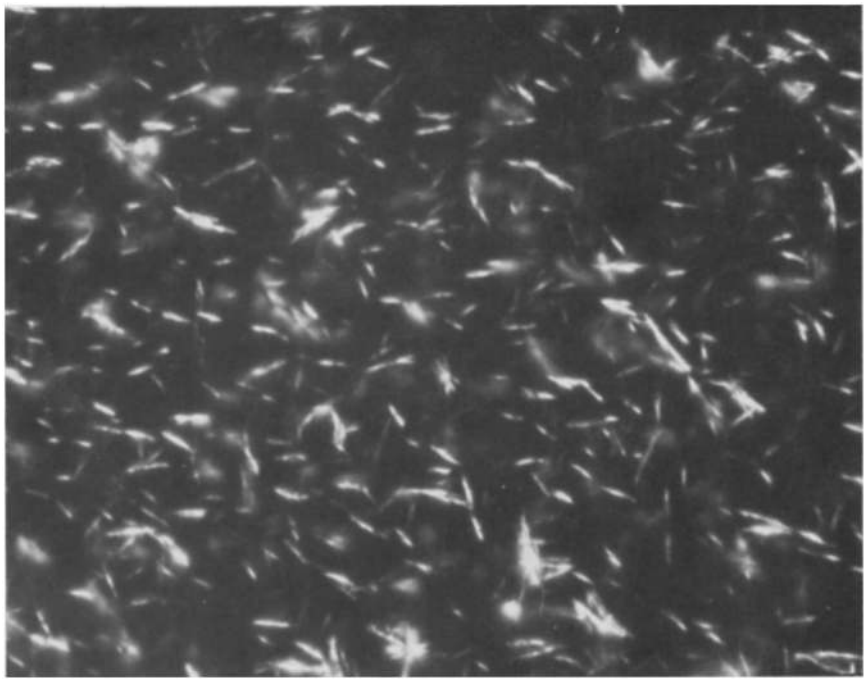

FIG. 2. Purified pili aggregates as viewed by darkfield illumination (× 1,800).

fields contain what appear to be only pili (Fig. 3), and by SDS-PAGE two protein-containing bands are seen after Coomasie Blue staining; a very heavily stained band which migrates at a mol wt of 17,500 daltons and a very weakly staining band corresponding to a mol wt of 38,000 daltons (Fig. 4). This latter protein is a major outer membrane protein of *E. coli*, also designated protein Ia (18). Further purification by isopycnic centrifugation in cesium chloride (pili band at a density of 1.299 g/cm^3) results in preparations containing a single protein band of a mol wt of approximately 17,500. Amino acid analysis of these purified pili indicates an unusually high proportion of nonpolar residues and the majority of the charged groups are acidic (Table I). The minimum mol wt as determined from the amino acid analysis was 17,099. No amino sugars or muramic acid was detectable on the amino acid analysis.

The isoelectric point (pI) of native pili cannot be determined in a polyacrylamide gel because they are too large to migrate into the gel even in the presence of 6 M urea. Pili which had first been reduced in dithiothreitol and then reacted with iodoacetamide, however, migrated into a 4% polyacrylamide gel. Three bands were seen in such gels corresponding to pIs of 4.5, 4.9, and 5.1. These differences in pI may be due to alteration in some pili molecules in the reduction procedure or may be due to the presence of other proteins. The latter is less likely since no other proteins are seen on SDS-PAGE. It is also possible that charge heterogeneity exists in the natural state.

The HA Reaction. Mixing purified pili with a 2% solution of guinea pig erythrocytes on a microscope slide resulted in marked agglutination in 30–60 s (Fig. 5). Pilated K12 *E. coli* rapidly agglutinated guinea pig erythrocytes, but

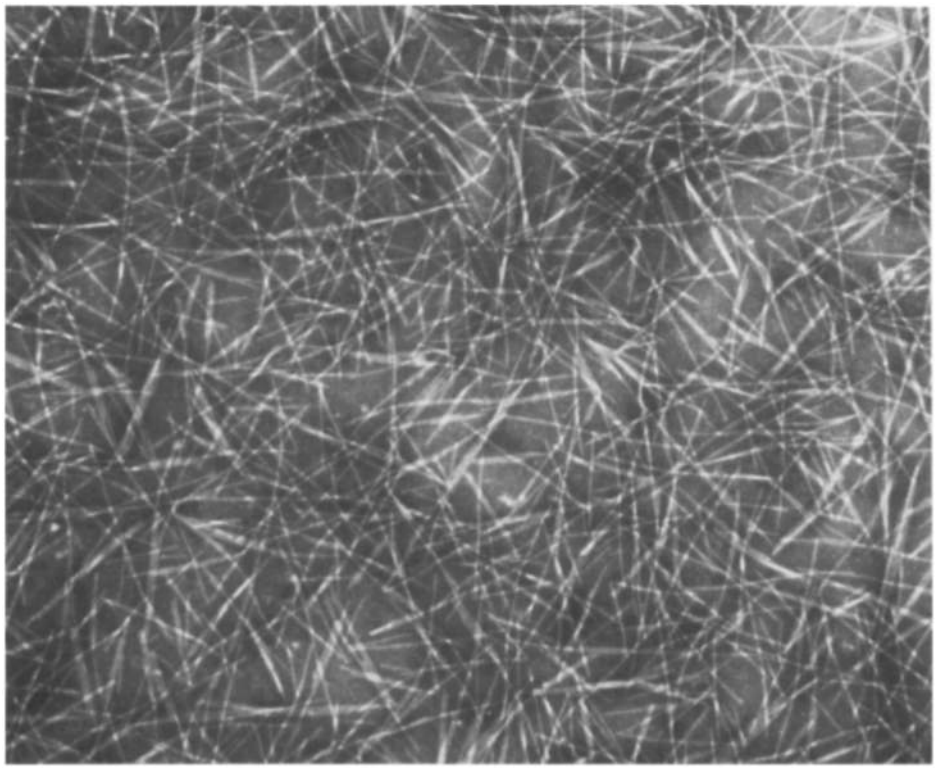

FIG. 3. Purified pili. Negatively stained with 2% PTA (× 48,000).

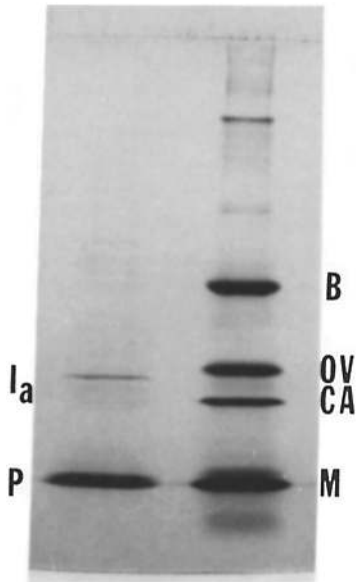

FIG. 4. SDS-PAGE of partially purified pili (12.5% gel). Gel purposely overloaded with 75 μg pili to detect contaminants. Stained with Coomassie Blue. Ia, protein Ia; P, pili. Molecular weight markers are BSA (B), ovalbumin (OV), carbonic anhydrase (CA), and sperm whale myoglobin (M).

TABLE I
Amino Acid Analysis of Type I E. coli Pili

Amino acid	No. of residues per mole	Integral no. of residues per mole
Nonpolar (45%)		
Alanine	34.0	34
Valine	14.0	14
Leucine	13.9	14
Isoleucine	5.0	5
Proline	2.1	2
Phenylalanine	8.4	8
Tryptophan	0	0
Methionine	0	0
Acidic (20%)		
Aspartic	17.7	18
Glutamic	16.0	16
Basic (5%)		
Lysine	4.4	4
Arginine	2.2	2
Histidine	2.1	2
Polar uncharged (30%)		
Glycine	20.6	21
Serine	9.3	9
Threonine	20.1	20
1/2 Cystine	2.0	2
Tyrosine	2.4	2
		Total 173

Calculated mol wt = 17,099.

the nonpilated variant did not cause HA even when 100-fold more viable organisms were present (Table II). As little as 3.3 μg/ml of purified pili resulted in unequivocal HA of the guinea pig cells. Irrespective of the ABO or Rh blood group, human cells were not readily agglutinated by pili unless the erythrocytes had been preincubated with trypsin. Similarly, pretreatment with trypsin increased the sensitivity to agglutination by Con A almost 10-fold (Table II).

The HA of guinea pig cells by purified pili was further studied under various conditions (Table III). No significant difference in the concentration of pili required for HA was found at 4°C, 24°C, and 37°C and pretreatment of erythrocytes with protease or trypsin had little effect on HA. Pretreatment with glycosidases used individually or together also had no significant effect on HA titers except α-mannosidase pretreatment which usually resulted in a twofold drop in titer when either pili, Con A, or *Lens culinaris* lectin was used as the hemagglutinin. Neuraminidase usually resulted in an increase in agglutinability.

The HA titers were unchanged by EDTA pretreatment of erythrocytes even if the HA were carried out in the presence of 50 mM EDTA. It is clear then that blood can be collected in EDTA without significant alteration of HA. Sonication of pili for 15 s at 50 W in a cell disruptor (Model 185; Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) broke up pili into small fragments and considerably reduced their ability to cause HA (Fig. 4 [well no. 6] and Table III).

Hapten Inhibition. Several sugars were found to be highly potent in pre-

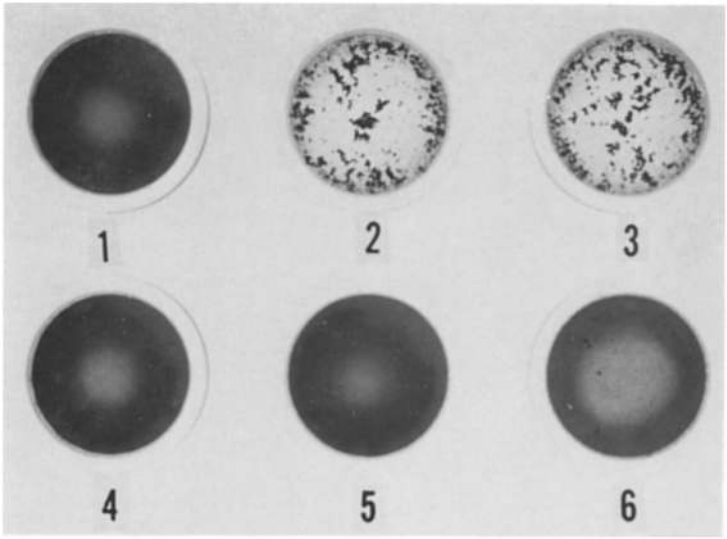

FIG. 5. Slide agglutination of guinea pig erythrocytes by purified pili (250 μg/ml). (1) Control – PBS, (2) pili, (3) pili plus 50 mM galactose, (4) pili plus 10 mM α-CH_3-mannoside, (5) pili plus 10 mM D-mannose, and (6) sonicated pili.

TABLE II
HA by Pili and Con A

	Red cells		
	Guinea pig	Human (untreated)	Human (trypsinized)
Con A, *μg/ml*‡	0.5 (± 0.2)*	58.2 (± 12.9)	5.9 (± 2.5)
Pilated K12, *cfu/ml*	2.4×10^6 ($\pm 0.82 \times 10^6$)	$>1.4 \times 10^6$	0.5×10^6 ($\pm 0.2 \times 10^6$)
Nonpilated K12, *cfu/ml*	$>2.5 \times 10^6$	$>2.5 \times 10^6$	$>2.5 \times 10^6$
Pili, *μg/ml*	3.6 (± 1.9)	>200	75 (± 27)

* Mean (± SD) of at least six determinations of minimal HA concentrations.
‡ Incubations done at 24°C.

venting the HA by pili (Table IV). The most potent inhibitors were AMM, D-mannose, and yeast mannan which is a highly branched mannose polymer containing α1–2 and α1–3 mannose-linked branches on a linear α1–6 linked backbone. The L isomer of mannose, however, was noninhibitory at a concentration of 100 mM. Similar results were found using this HA system but substituting *Lens culinaris* or Con A as the hemagglutinins.

Anti-Pili Antibodies. Antibodies raised against purified pili inhibited HA when diluted 1:8. Anti-P+ antisera were inhibitory at 1:1 and anti-P− antisera were not inhibitory. Anti-P+ antisera did contain anti-pili antibodies and anti-P− antisera did not contain anti-pili antibodies as detected by precipitin reaction in agar using purified pili.

Discussion

The isolation of type I pili from *E. coli* is often complicated by the co-isolation of other bacterial appendages such as sex pili and flagellae and by components of the outer membrane. By using a bacterial variant deficient in sex pili and

TABLE III
HA of Guinea Pig Cells by Purified Pili

Hemagglutination conditions	Concentration of pili required for HA
	μg/ml
Temperature 37°C	3.9 (± 1.3)*
24°C	3.6 (± 1.2)
4°C	3.6 (± 0.65)
Trypsin (200 μg/ml)	4.9 (± 1.6)
Protease (1,000 μg/ml)	3.9 (± 1.3)
α-Galactosidase (1 U/ml)	3.3 (± 0)
β-Galactosidase (1 U/ml)	3.9 (± 1.3)
β-Glucosidase (1 U/ml)	3.9 (± 1.3)
α-Mannosidase (1 U/ml)	7.1 (± 3.2)
Mixed glycosidases (6.25 mg/ml)	3.8 (± 0.82)
Neuraminidase (0.6 U/ml)	1.9 (± 0.85)‡
EDTA (50 mM)	3.3 (± 1.1)
Sonicated pili	100.3 (± 11.8)§

* Mean (SD) of six determinations.
‡ Comparison made to incubated control (P = <0.05) (Student's t test).
§ Comparison made to incubated control (P = <0.01) (Student's t test).

TABLE IV
Inhibition of Pili HA by Saccharides

Saccharide	Concentration required to inhibit four MHC
	mM
α-CH_3-D-mannoside	1.5
D-Mannose	2.3
Yeast mannan	4.1
D-Fructose	11.3
N-acetyl mannosamine	75.0
D-Glucose	>100*
α-CH_3-D-glucose	>100
L-Mannose	>100
D-Fucose	>100
L-Fucose	>100
Sucrose	>100
D-Galactose	>100
Lactose	>100
Mannitol	>100
Maltose	>100
D-Arabinose	>100

* No inhibition at 100 mM.

flagellae (8), one can obtain, using precipitation by ammonium sulfate, a large yield of partially purified type I pili. For some studies, for example, investigation of pili composition, we have found further purification procedures necessary to remove contaminating outer membrane protein.

The characteristics of pili isolated by this procedure closely resemble those described by Brinton (8). The molecular weight of pili purified by our method is not significantly different from that previously reported. The results we have obtained from amino acid analysis differ in that we find more glycine, leucine,

and glutamic acid and less aspartic acid. Morphologically the pili appear identical in size and fine structure.

The pilated variant of K12 is at least a 100-fold more potent agglutinator of guinea pig erythrocytes as compared to the nonpilated variant, but other differences in these bacteria besides pilation could conceivably lead to such results. The use of purified pili however, confirmed that the HA is due to the pili alone. Although the mode of such agglutination is unclear, it is likely that a specific recognition binding event occurs akin to lectin binding of sugar molecules (19) since saccharides possessing only a limited range of configurations inhibit binding.

The results of our hapten inhibition studies confirm the findings previously reported for Salmonella and Shigella bacteria HA by Old (20). The most potent inhibitors in both studies are D-mannose, AMM, D-fructose, and yeast mannan. Presumably, such saccharides resemble or in some cases are identical to residues available for binding of pili on the mammalian cell membrane. The most effective inhibitors of pili HA are also the most effective inhibitors of Con A HA, and we have found a general correlation between Con A agglutinability and pili agglutinability of erythrocytes from several tested species (man, guinea pig, and sheep).

The bulk of pili binding could not be eliminated by treatment with EDTA, glycosidases, or proteolytic agents. Mild trypsinization or treatment with commercial protease mixtures in fact enhances agglutination of human erythrocytes. This phenomenon, also noted for Con A agglutination of erythrocytes (21), has been attributed to increased receptor mobility secondary to loss of spectrin and is correlated with clustering of protein particles as viewed by freeze fracture (22). Trypsinization causes loss of the major glycoprotein of the human erythrocyte (PAS1) (23), and EDTA can result in elution of bands I and II (24) and it is likely the pili-binding site does not reside in these eluted fractions. Con A receptors are believed to reside in band III of the erythrocyte (25) which is partially broken down by proteases but may not be completely lost (23).

Among the many factors important is cell agglutination by lectins is the effect of cell surface charge density (26). By releasing cell membrane sialic acid through proteolytic digestion or neuraminidase treatment, lectin agglutination is enhanced (27). Our results with pili HA are consistent with those findings.

Certain lectins are known to interact at core sites of oligosaccharides in addition to their binding of terminal residues, and it is possible pili do so also. α-Mannosidase pretreatment of erythrocytes results in a slight reduction in agglutinability which could be explained by release of the terminal mannose residues important in Con A and pili binding. A commercial mixture of glycosidases (containing α-mannosidase but without neuraminidase) had less effect on agglutination, but contaminating proteases make these results difficult to interpret. If mannose is the key determinant of the pili-binding site, it is probably in a nonterminal position and hence inaccessible to these enzymes. In most defined glycoproteins containing mannose, this molecule is located at branch points, but since surface glycoproteins exist in various degrees of completion, it is likely there always exists some terminal mannose. The release of such molecules may be responsible for the observed action of α-mannosidase.

Con A agglutination of human erythrocytes is a temperature-sensitive phenomenon (21). Although proteins in a lipid bilayer are relatively immobile below 15°C (as measured by antigen admixture experiments) (28), pili agglutination was identical at 4°C and 37°C, and we have found similar results when pili binding to monolayers of tissue culture cells is measured (29) indicating a lack of importance of membrane fluidity in pili binding.

Certain other differences between pili and Con A HA exist such as differences in hapten inhibition particularly with respect to glucose inhibition (30). Also, the reported enhancement of Con A HA by adenosine (31) does not occur with pili. It seems likely that the groups of molecules to which Con A binds may be of wider specificity than for pili. There is evidence that Con A binding may include the pili-binding site since when the lectin binds to tissue cells, it can inhibit pili binding (29).

Summary

Many enterobacteria can cause agglutination of erythrocytes, but previous investigations have not proven which components of the bacteria are responsible. We used a strain of *Escherichia coli* K12 which causes mannose-sensitive hemagglutination (HA) of guinea pig cells. Common pili were purified from these bacteria by shearing them from the bacteria followed by selective precipitation in acid and ammonium sulfate. Isopycnic centrifugation in cesium chloride removed the remaining outer membrane protein contaminants. These pili are pure by electron microscopy and gel electrophoresis. By amino acid analysis, they have a mol wt of 17,099 and consist of 45% nonpolar residues. These purified pili agglutinate guinea pig erythrocytes, a reaction that is inhibited by anti-pili antibodies and by saccharides related in structure to D-mannose. Proteolytic treatment of erythrocytes does not diminish HA but rather increases the pili-induced HA of human cells. Neuraminidase enhances HA and mannosidase slightly diminishes it. It is concluded that purified pili alone cause HA of erythrocytes by binding to mannose-like molecules on the erythrocyte surface. Thus HA by bacterial pili serves as a useful model system for the mechanism of bacterial pili attachment to cell membranes.

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