INFECTION AND IMMUNITY, Nov. 1978, p. 462-472
0019-9567/78/0022-0462$02.00/0

Vol. 22, No. 2
Printed in U.S.A.

Differences in Hydrophobic Surface Characteristics of Porcine Enteropathogenic *Escherichia coli* With or Without K88 Antigen as Revealed by Hydrophobic Interaction Chromatography

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Received for publication 10 July 1978

Porcine enteropathogenic *Escherichia coli* strains possessing or lacking K88 antigen were studied by using hydrophobic interaction chromatography on cross-linked agarose gels with alkyl or aryl substituents (amphiphilic gels) to determine whether or not they possessed surface-associated hydrophobic properties. Strains with K88ab or K88ac antigen adsorbed to phenyl and octyl Sepharose gels in the presence of 4 M sodium chloride. This property correlated with phenotypic expression of K88 antigen. Cells grown at 37°C but not those grown at 18°C possessed hydrophobic adsorptive characteristics in addition to the property of mannose-resistant hemagglutination of guinea pig erythrocytes. Adsorption of K88-positive strains to gels with hydrophobic ligands was independent of O group and enterotoxicity. Strains lacking K88 antigen did not adsorb to the hydrophobically substituted derivatives of Sepharose and lacked mannose-resistant hemagglutinating characteristics. Neither the presence of additional polysaccharide K antigens nor nonhemagglutinating pili conferred the property of hydrophobic interaction on the strains. K88-positive bacteria had a lower electrophoretic migration rate than did K88-negative bacteria of the same serotype in free-zone electrophoresis. K88-positive bacteria also adsorbed strongly to hydrophobic ligands in the presence of 1 M ammonium sulfate, whereas K88-negative strains did not. These observations provide evidence for the suspected role of hydrophobic interaction in the adhesive properties of certain enteropathogenic strains of *E. coli*. Moreover, hydrophobic interaction chromatography provides convenient and rapid alternative means of screening strains for a property potentially associated with adhesiveness.

In recent years, the adhesive properties of bacteria towards mucosal membranes, in particular toward intestinal mucosa, have received much attention in relation to bacterial pathogenicity (1, 21). Colonization of the small intestine of piglets, calves, lambs, and humans (both infants and adults) by enteropathogenic *Escherichia coli* has been associated with the possession of pilus-like adhesins on the surfaces of these bacteria; these are referred to as K88 antigen, K99 antigen, and colonization factor (6, 7, 10, 20, 29). Nonhemagglutinating pili and common type 1 pili may also mediate adherence in certain instances (19, 37).

In only a few instances, by the use of lectins and by modification of cell surface charge, have eucaryotic cell surface receptors for bacteria been investigated and characterized by tests for the inhibition of hemagglutination with monosaccharides or glycoproteins (20, 38). Biophysical characteristics that may possibly influence bacterial adhesion to eucaryotic cells have been reviewed by Jones (20).

Studies on bacterial cell surface characteristics such as charge and hydrophobic properties have provided valuable insights on the nature of molecular groupings involved in the interaction of bacteria with host cell membranes. Partition between various aqueous polymer two-phase systems and free-zone electrophoresis have been used to investigate physiochemical properties of the cell surface of smooth and rough *Salmonella typhimurium* and of *E. coli* (25, 26, 45, 46), and the observed surface charge densities and hydrophobicities have been correlated with liability to phagocytosis (47, 49) and with attachment to

intestinal mucosa (32). Lipophilic strains of *S. typhimurium* and *E. coli* showed a greater association with intestinal mucosa than more hydrophilic strains. Isoelectric focusing has also been used to study surface charge properties (13, 41).

Chromatographic procedures have provided information of relevance to adherence. Hall et al. (11) fractionated *S. typhimurium* cells by ion-exchange chromatography into two populations and could differentiate *S. typhimurium* from *E. coli* on the basis of surface charge properties. Moreover, the binding of oral streptococci to hydroxyapatite has revealed differing affinities and binding sites for these closely related species (23) and has indicated that electrostatic forces may be involved in their interaction with tooth surfaces (34).

Hydrophobic interaction chromatography (HIC) is a chromatographic procedure based on hydrophobic interaction between nonpolar groups on a gel bed and nonpolar regions of a solute, e.g., of a protein (16). Adsorbents with alkyl or aryl substituents have been synthesized by coupling reactions to agarose gels (17, 35, 40). The degree of hydrophobicity of the substituents and the degree of substitution can be varied (36). (Although, strictly speaking, they are amphiphilic or amphipathic gels, the derivatized Sepharose gels with hydrophobic substituents used herein will be referred to as "hydrophobic gels.")

HIC has been applied to the purification of satellite tobacco necrosis virus and the fractionation of yeast cells (17). Furthermore, alkyl agaroses have been reported to exhibit different adsorption profiles for different erythrocyte species (12). These studies with hydrophobic gels suggested the possibility that HIC might provide a simple and useful means of discriminating between bacterial strains with respect to the hydrophobicity of their surfaces or adhesins. Because the amino acid composition of K88 antigen (19, 48) revealed a preponderance of amino acids with apolar side chains, studies on the hydrophobicity of porcine enteropathogenic *E. coli* strains possessing or lacking K88 antigen were undertaken by using HIC. The data indicate that possession of pilus-like, surface-associated antigens by enteropathogenic *E. coli* confers hydrophobic characterisitics that are likely to provide the driving force for procaryotic-eucaryotic cell surface interaction through displacement of water from the interacting surfaces, and the formation of an adhesive bond through a gain in entropy.

MATERIALS AND METHODS

Chemicals. Phenyl Sepharose CL-4B (lot. no. 9705; degree of substitution = 0.23 mol of ligand per mol of galactose), octyl Sepharose CL-4B (lot no. 9080; degree of substitution = 0.16) and Sepharose CL-4B (lot no. 8932) were purchased form Pharmacia AB, Uppsala, Sweden. D-Mannose was obtained from E. Merck, Darmstadt, West Germany. All other chemicals were of analytical grade or of the best grade commercially available.

Synthesis of hydrophobic agarose gels. (i) Palmitoyl Sepharose CL-4B (lot no. 98620; degree of substitution = 0.13). A 500-ml amount of sedimented Sepharose CL-4B was transferred to a glass filter. Solvent exchange to 1,2-dichloroethane was performed by the method of Hjertén et al. (17). The suspension, consisting of 750 ml of gel and 1,2-dichloroethane was poured into a three-necked, round-bottomed flask fitted with a condenser, stirrer, and funnel. Pyridine (8 ml) was added, and the mixture was gently stirred. Palmitoyl chloride (29.5 ml), dissolved in 150 ml of 1,2-dichloroethane, was added over a period of 1 h. The reaction mixture was kept at 60°C for another 2 h. It was then poured into a glass filter funnel and washed with 500-ml portions of 1,2-dichloroethane (twice), acetone (six times) and, finally, water (six times). The degree of substitution was determined by nuclear magnetic resonance (35).

(ii) Naphthoyl Sepharose CL-6B (400 ml; lot no. 97714 A; degree of substitution = 0.15). Sepharose CL-6B was transferred to a glass filter funnel and then washed with acetone (400 ml; six times) and 1,2-dichloroethane (400 ml; six times). The gel was not allowed to become surface dry between the different washings. The gel suspension was then transferred to a reaction vessel of the same kind as for palmitoyl Sepharose, and the reaction performed under similar conditions. Pyridine (25 ml) and 1-naphthoyl chloride (40 ml) were used. After the coupling reaction, the gel was washed on a filter with acetone (400 ml; six times). The final washings were done with water (2,500 ml; five times). The degree of substitution was determined by nuclear magnetic resonance (35).

(iii) Dodecyl and naphthyl Sepharose. Dodecyl Sepharose (lot no. 257814; degree of substitution = 0.06) and naphthyl Sepharose (lot no. 97712 A; degree of substitution = 0.13) were synthesized by O alkylation and O arylation, respectively, of Sepharose CL-4B in 1,2-dichloroethane (17). Degrees of substitution were determined by nuclear magnetic resonance (35).

Bacterial strains. The various *E. coli* strains studied are described with respect to origin, serotype, and enterotoxicity in Table 1. Strains originating from the National Veterinary Institute, Stockholm, were isolated from piglets between 1975 and 1977. Strains were preserved in Trypticase soy broth (Baltimore Biological Labortory; Cockeysville, Md.) containing 15% (wt/vol) glycerol at −70°C. After one subculture, strains received from abroad were preserved similarly.

Serology. O-group determination of Swedish strains was performed by the method of Söderlind (42), using antisera to the 15 most common O groups encountered in piglets with colibacillosis in Sweden (O groups 2, 6, 8, 9, 32, 45, 64, 98, 115, 138, 139, 141, 147, 149, and 157). Strains which did not belong to these O groups were sent to the International Escherichia Center, Copenhagen, for O grouping. The O groups of strains from the Netherlands, the United States and Denmark were accepted as designated by

TABLE 1. *Characteristics of Escherichia coli examined by hydrophobic interaction chromatography*

Group	K antigen	O group	No. of strains or strain designation	Other characteristics[a]
I (Phenyl)[b]	K88$^-$	8	4	ST$^-$LT$^-$ (3); ST$^+$LT$^-$ (1)
		9	1	ST$^-$LT$^-$
		20[c]	1	ST$^-$LT$^-$
		64	2	ST$^+$LT$^-$
		101[c]	1	ST$^-$LT$^-$
		107[c]	1	ST$^-$LT$^-$
		115	1	ST$^-$LT$^-$
		118[c]	1	ST$^-$LT$^-$
		139	1	ST$^-$LT$^-$
		157	1	ST$^-$LT$^-$
		Neg[c,d]	2	ST$^-$LT$^-$; ST$^+$LT$^-$
		Auto[e]	1	ST$^-$LT$^-$
	K88$^+$	149	10	ST$^+$LT$^+$ (6); ST$^-$LT$^+$ (4)
II (Octyl)[b]	K88$^-$	1[c]	1	ST$^-$LT$^-$
		2	1	ST$^-$LT$^-$
		6	1	ST$^-$LT$^-$
		9	3	ST$^-$LT$^-$ (1); ST$^+$LT$^-$ (2)
		20:54[c]	1	ST$^-$LT$^-$
		64	1	ST$^-$LT$^-$
		77[c]	1	ST$^-$LT$^-$
		82[c]	1	ST$^-$LT$^-$
		98	1	ST$^-$LT$^-$
		112[c]	2	ST$^-$LT$^-$
		115	2	ST$^-$LT$^-$
		119[c]	1	ST$^-$LT$^-$
		139	2	ST$^-$LT$^-$
		140[c]	1	ST$^+$LT$^-$
		141	1	ST$^-$LT$^+$
		149	1	ST$^+$LT$^+$
		Neg[c,d]	2	ST$^-$LT$^-$
		OX 8[c]	1	ST$^-$LT$^-$
	K88$^+$	141	1	ST$^+$LT$^+$
		149	8	ST$^+$LT$^+$ (3); ST$^-$LT$^+$ (5)
III[f,g]	K?:K88ac[h]	8	1	*ST$^?$LT$^+$*
	K?:K88ab	8	1	ST$^-$LT$^+$
	K87:K88ab	8	3	ST$^+$LT$^+$ (2); ST$^-$LT$^+$ (1)
	K87:K88ac	8	3	ST$^-$LT$^+$ (1); *ST$^?$LT$^+$* (2)
	K(A)2347:K88ab	9	3	ST$^+$LT$^+$
	K?:K88ac	20	1	ST$^-$LT$^+$
	K$^-$:K88ac[h]	45	3	ST$^-$LT$^+$
	K81:K88ac	138	3	ST$^-$LT$^+$
	K89:K88ac	147	3	ST$^+$LT$^+$ (2); *ST$^?$LT$^+$* (1)
	K$^-$:K88ac	157	3	ST$^-$LT$^+$ (1); *ST$^?$LT$^+$* (2)
	K87	8	3	ST$^-$LT$^+$
	K$^-$	45	3	ST$^-$LT$^-$
	K81	138	3	*ST$^?$LT$^+$*
	K$^-$	157	3	ST$^-$LT$^+$ (2); *ST$^?$LT$^+$* (1)
IV[i]	K$^-$:K88ac	147	G 1253	H19
	K$^-$	147	D 357	H19
V[j]	K(A)103	9	987	Wild type[k], K$^+$P$^+$
	K(A)$^-$	9	I 27	K$^-$P$^-$
	K(A)103	9	I 36	K$^+$P$^-$
	K(A)$^-$	9	I 40	K$^-$P$^+$
		9	I 50	
VI[l]	K88$^-$K99$^-$		K-12	Wild type
	K88ab		K-12	K-12 (K88)

[a] ST, Heat-stable enterotoxin, production assayed in suckling 3-day-old mouse test by the method of Giannella (8); ST$^+$, intestine to body weight ratio ≥ 0.09; ST$^-$, intestine to body weight ratio < 0.08; LT, heat-labile enterotoxin, production assayed in the mouse Y1 adrenal cell test of Donta et al. (5): LT$^+$, ≥25% rounding off

TABLE 1—*Continued*

of the adrenal cells; LT⁻, <25% rounding off of the adrenal cells; *ST'*, heat-stable enterotoxin not determined; *LT*⁺, production of heat-labile enterotoxin by the Vero cell test (44). Number of strains given in parentheses where appropriate.

[b] Strains from National Veterinary Institute, Stockholm, Sweden.

[c] Strains serotyped at the International Escherichia Center, Copenhagen, Denmark.

[d] Not typable by antisera to O groups 1 to 165.

[e] Autoagglutinating, rough strain.

[f] Strains from P. A. M. Guinée, National Institute of Public Health, Bilthoven, Netherlands.

[g] Data on enterotoxin production kindly supplied by P. A. M. Guinée.

[h] K⁻, No K antigen of polysaccharide type present; K?, K antigen nontypable.

[i] Strains from F. Ørskov and I. Ørskov, Statens Seruminstitut, Copenhagen, Denmark.

[j] Strains from H. W. Moon, National Animal Disease Center, Ames, Iowa.

[k] See reference 19 for details.

[l] Strains from C. Gyles, University of Guelph, Guelph, Ontario, Canada.

the donors. K typing, excluding tests for K88 antigen, was only performed on a few of the Swedish porcine isolates (data not shown); the data on additional K antigens of the Dutch strains are those received from P. A. M. Guinée (Table 1). Possession of K88 antigen by Swedish strains was determined by a slide agglutination test (42), using specific anti-K88 antiserum (see below); strains grown at 37°C which gave positive agglutination reactions were retested after growth at 18°C (22, 31). K88 ab and ac designations of Dutch and Danish strains are those supplied by the respective donors. In addition, all strains used in this investigation were rechecked independently at Biomedicum, Uppsala, Sweden, for the presence or absence of K88 antigen by using an absorbed K88 antiserum (see below) and without knowledge of the original K88 designations of strains.

K88 antiserum. New Zealand white rabbits were given initially three injections (0.5 ml, 3-day intervals) followed by three injections (1 ml, weekly intervals) of an experimental "piglet enteritis vaccine" comprising killed bacteria (vaccine A; *E. coli* O149:K91, K88ac, K88ab:H10) kindly supplied by I. Nagy, Wellcome Research Laboratories, Beckenham, England. Antiserum pools were absorbed with an O149:K91:H10 strain using both Formalin-treated and heat-killed cells and with five other K88-negative *E. coli* strains which gave weak (nonspecific) agglutination reactions with undiluted absorbed serum. Absorbed antiserum was precipitated with ammonium sulfate (35% saturation), and the immunoglobulin fraction was dissolved in 0.15 M sodium chloride to the original serum volume. After dialysis against 0.15 M sodium chloride containing 15 mM sodium azide, the antibodies were stored at 4°C.

Growth conditions and bacterial suspensions for HIC. Strains were grown in 100 ml of a tryptone—yeast extract medium (TY-1) which contains 1% (wt/vol) glucose (18) in 1-liter indented shake flasks (120 rpm; 37°C, for 1 to 2 h) to an absorbance at 600 nm (A_{600}) of ≥ in a 10-mm light path, corresponding to about 1.4×10^9 colony-forming units (CFU) per ml. Inocula comprised 10 ml of 16- to 18-h cultures grown under identical growth conditions in TY-1 medium. Portions of test cultures (20 ml) were centrifuged (4,000 × *g*; 10 min; 20°C), and cell pellets were gently suspended in 4 M sodium chloride or 1 M ammonium sulfate buffered with 10 mM sodium phosphate buffer (pH 6.8) to obtain homogeneous suspensions with about 2×10^{10} to 4×10^{10} bacteria per ml for HIC.

A number of selected strains, representative of the serotypes in the K88 material, were grown on glucose-nutrient agar (21) at 37°C for 18 h and at 18°C for 48 h. Bacteria from agar plates were suspended directly in buffered 4 M sodium chloride by using cotton swabs, and bacterial concentrations were adjusted as described above. Strains were also grown on heart infusion agar (Difco Laboratories, Detroit, Mich.) at 37°C for 18 h, and suspensions were prepared as described above.

HIC. Hydrophobic derivatives of Sepharose were washed extensively with buffered 4 M sodium chloride or 1 M ammonium sulfate (see above) to remove fine particles and sodium azide or ethanol added to the agarose gel suspensions as preservative (33). Gel suspensions were allowed to equilibrate at 20 to 24°C, and chromatography was performed at the same temperature (16). Columns comprised short-ended glass Pasteur pipettes (internal diameter, 5 mm; length, 85 mm), plugged with a little glass wool and fitted with clamped Teflon tubing. For most experiments, gel beds were packed to a height of 30 mm (ca. 0.6 ml gel bed volume) by gravity feed and washed with 10 ml of buffered 4 M sodium chloride or 1 M ammonium sulfate. Bacterial suspensions (100 μl, 2×10^9 to 4×10^9 bacteria) were allowed to drain into the gel beds. Gel beds were washed with 5 ml of buffered 4 M sodium chloride or 1 M ammonium sulfate (flow rate, 1 to 2 ml/min). For routine purposes (e.g., screening of hydrophobicity of strains), visual comparison of the opacity of the eluate with an appropiately diluted 100-μl portion of the original suspension was used to assess the degree of adsorption. Visible adsorption to the gel bed and its appearance were recorded (Fig. 1). All strains were run "blind" as coded, randomized samples, with the inclusion of adsorbing and nonadsorbing strains as controls.

Release of bacteria that adsorbed to hydrophobic gels was attempted in certain experiments by decreasing the ionic strength (16). Therefore, desorption was performed by washing the gel bed with 10 ml of 10 mM sodium phosphate buffer (pH 6.8). The turbidities of eluates were again compared visually with appropriately diluted portions of the original suspensions. Adsorption and desorption were also quantified by viable counts and bacterial counts related to measure-

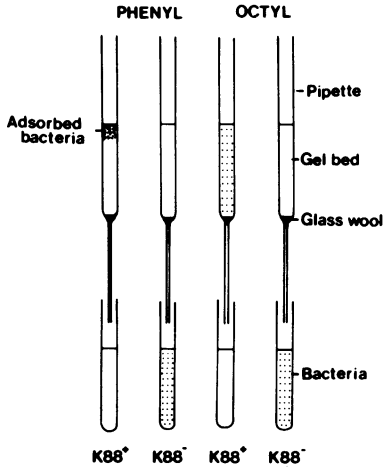

FIG. 1. *Diagrammatic representation of the appearance of gel beds upon HIC of K88-positive and K88-negative strains of E. coli on phenyl and octyl Sepharose in the presence of buffered 4 M NaCl at 20°C.*

ments of A_{600} of suspensions in a 10-mm light path.

Chromatography on Sepharose CL-4B. Chromatography was also performed on Sepharose CL-4B with no ligand attached under the conditions described above to control for possible nonspecific adsorption effects on the Sepharose gel beads.

Viable counts. Bacterial suspensions were diluted ten-fold in 0.15 M sodium chloride buffered with 10 mM sodium phosphate buffer (pH 6.8). Eighteen 25-μl drops (MLA micropipette, Vernon, N.Y., with sterile disposable tips) for each appropriate dilution were applied to surface-dried heart infusion agar plates (six per plate), and CFU were counted after incubation at 37°C for 24 h.

Determination of bacterial numbers by absorbance measurements. Standard curves were constructed relating measurements of A_{600} in a 10-mm light path to CFU of bacteria per ml. This was done by using strain G 1253 (Table 1) grown to mid-logarithmic phase in TY-1 medium as described above. It must be emphasized that absorbance measurements increase markedly for identical suspensions of both K88-positive and K88-negative *E. coli* in concentrations of sodium chloride >0.3 M. It is, thus, important to dilute suspensions or column eluates with the diluent or eluant relevant to particular experiments.

Mannose-resistant hemagglutination test. Bacterial suspensions for hemagglutination assays were prepared from TY-1 broth cultures, glucose-nutrient agar and heart infusion agar similarly to those for HIC studies, except that cells were suspended in ice-cold 0.15 M sodium chloride (pH 6.8). Twofold dilution series of bacterial suspensions were prepared in 0.15 M sodium chloride containing 0.5% (wt/vol) D-mannose using plastic Microtiter trays (Flow Laboratories, Irvine, Scotland) essentially by the method of Jones and Rutter (22). Fresh guinea pig erythrocytes (3% vol/vol with respect to initial blood volume), washed three times, were used for detection of K88 antigen-mediated hemagglutination. Titrations were incubated on ice for 1 h (22). Titrations were performed blind on coded, randomized bacterial suspensions. All K88 titrations included known positive and negative strains as controls.

Free-zone electrophoresis. Electrophoresis experiments were performed in a Hjertén apparatus which consists of a horizontal quartz tube slowly rotating around its long axis (15). Logarithmic growth phase bacteria were centrifuged from TY-1 medium and resuspended in 0.4 M sucrose buffered with 30 mM sodium phosphate buffer (pH 6.8). Electrophoretic migration rate was recorded by UV scanning at 10-min intervals (15).

RESULTS

Hydrophobic interaction chromatography of paired K88-positive and K88-negative *E. coli*. Suspensions of the paired strains G1253(K88ac) and D357($K88^-$) and K-12(K88ab) and K-12($K88^-K99^-$) (groups IV and VI, respectively, in Table 1) were chromatographed on phenyl and octyl Sepharose in the presence of 4 M sodium chloride. Typical experiments are illustrated in Fig. 1. In each case, the K88-positive member of each pair of strains strongly adsorbed to the hydrophobic ligands, whereas the K88-negative bacteria did not appear to adsorb to either gel. With phenyl Sepharose, the K88-positive bacteria adsorbed as tight bands at the tops of the gel beds. In contrast, with octyl Sepharose, they adsorbed throughout the gel beds. Wash eluates of the hydrophobic gels to which K88-positive bacteria had adsorbed with buffered 4 M sodium chloride were of negligible turbidity ($\leq 10^6$ bacteria/ml).

Representative quantitative adsorption data from duplicate columns, using viable counts for one of these paired strains, are shown in Table 2. Phenyl Sepharose appeared to have a higher adsorptive capacity than octyl Sepharose in the presence of 4 M sodium chloride. Similar observations were obtained with strains G1253 and D357. The viability of logarithmic phase cells from TY-1 medium in 4 M NaCl did not drop significantly over a 2-h period at room temperature. All HIC experiments were performed well within this time interval. Although viable counts of identically prepared suspensions in 0.15 and 4 M sodium chloride were usually ≤25% higher in 0.15 sodium chloride in parallel experiments, on no occasion were differences in initial viable counts statistically significant.

K88-positive bacteria were not readily desorbed from these hydrophobic gels by lowering of the ionic strength (Table 2). There was no loss in the viability of *E. coli* cells in 4 M sodium chloride upon sudden dilution in 10 mM sodium phosphate buffer (pH 6.8) compared with cells diluted in 0.15 M sodium chloride or cells diluted in decreasing concentrations (2, 1, 0.5, 0.25, and

TABLE 2. *Quantitative data on adsorption to hydrophobic gels based on viable counts of a K88⁺ and K88⁻ pair of strains of E. coli*[a]

Strain	Fraction	Total viable counts of bacteria (CFU) with:	
		Phenyl Sepharose	Octyl Sepharose
K-12 (K88ab)	Applied sample	$(2.95 \pm 1.06) \times 10^9$	$(2.95 \pm 1.06) \times 10^9$
	Buffered 4 M NaCl wash[b]	$(5.42 \pm 1.74) \times 10^5$	$(1.23 \pm 0.25) \times 10^7$
	Buffer desorption eluate[c]	$(3.07 \pm 0.99) \times 10^5$	$(2.22 \pm 1.20) \times 10^5$
K-12(K88⁻K99⁻)	Applied sample[d]	$(2.36 \pm 1.04) \times 10^9$	$(2.36 \pm 1.04) \times 10^9$
	Buffered 4 M NaCl wash[b]	$(1.88 \pm 0.31) \times 10^9$	$(1.95 \pm 0.42) \times 10^9$
	Buffer desorption eluate[c]	$(0.15 \pm 0.07) \times 10^9$	$(0.27 \pm 0.16) \times 10^9$

[a] In these experiments, autoclaved Pasteur pipettes, glass wool, 4 M NaCl, desorption buffer, and collection tubes were used. Phenyl and octyl Sepharoses as supplied by the manufacturer contained preservatives and were extensively washed with sterile 4 M NaCl before use; all manipulations were performed as aseptically as possible; control columns washed with sterile 4 M NaCl did not reveal >10 CFU of bacteria per ml of wash (*Bacillus* sp. by colony morphology and Gram stain); any non-*E. coli* colonies by morphology were ignored when determining CFU. of *E. coli*.

[b] Wash out of applied sample with 10 ml of 4 M NaCl containing 10 mM sodium phosphate buffer (pH 6.8).

[c] Eluate obtained upon desorption of adsorbed applied sample as described in footnote *b* with 10 mM sodium phosphate buffer (pH 6.8).

[d] The differences in viable counts in the applied samples to both phenyl and octyl Sepharoses and the viable counts in the 4 M NaCl washes are not statistically significant by a two-tail Student's *t* test.

0.15 M) of sodium chloride. K88-negative bacteria did not adsorb to phenyl or octyl Sepharoses within the limits of accuracy of the viable-counting method.

Analysis of Swedish porcine enteropathogenic *E. coli*. Groups I and II (Table 1) were examined by HIC on phenyl and octyl Sepharoses, respectively. Adsorption to hydrophobically substituted Sepharoses correlated well with the property of mannose-resistant hemagglutination of guinea pig erythrocytes and K88 antigen reactivity serologically (Table 3). All strains with K88 antigen adsorbed to the hydrophobic gels and caused mannose-resistant hemagglutination. The converse was true for the K88-negative strains.

Seven K88-positive and K88-negative (group I) and five K88-positive and K88-negative (group II) were run on octyl and phenyl Sepharose, respectively, as cross-checks between groups. All strains which adsorbed to phenyl Sepharose also adsorbed to octyl Sepharose and vice versa. All K88-negative strains failed to adsorb to both hydrophobic gels. Hydrophobic interaction did not correlate with heat-labile or heat-stable enterotoxin production.

Analysis of Dutch porcine enteropathogenic *E. coli*. Because of the high frequency of K88-positive strains within O group 149 among enteropathogenic *E. coli* isolates in Sweden (42, 43) (Table 1), the possibility existed that adsorption to hydrophobically substituted Sepharoses was reflective of O-group specificity rather than of K88 antigen and, thus, that the apparent correlation between adsorptive properties to hydrophobic gels, mannose-resistant hemagglutination, and the possession of K88 antigen was coincidental.

TABLE 3. *Hydrophobic adsorption, mannose-resistant hemagglutination, and K88 antigen reactivity of Swedish porcine enteropathogenic E. coli*

Strains[a]	Adsorption in HIC	MRHA[b]	K88 reactivity[c]
Group I K88⁺	+	+	+
I K88⁻	−	−	−
Group II K88⁺	+	+	+
II K88⁻	−	−	−

[a] See Table 1.

[b] MRHA, Mannose-resistant hemagglutination of guinea pig erythrocytes; titers ≥1:256 were obtained with all K88-positive strains (see text).

[c] Determined by agglutination test with K88 antiserum described in text. Results were in agreement with original designations in Table 1.

A series of strains from Holland was thus examined to clarify this question. Strains included serologically paired K88-positive and K88-negative strains within O groups 8, 45, 138, and 157. K88-positive strains of three O groups represented in the K88-negative material from Sweden were also tested, namely O groups 8, 9, and 20. The Dutch strains also possessed or lacked K antigens in addition to K88 antigen, e.g., K87, K81 and K⁻ (30). Irrespective of O serotype, K88-positive strains adsorbed to phenyl Sepharose in the presence of buffered 4 M sodium chloride, whereas K88-negative strains with or without other K antigens did not. All K88-positive strains also produced mannose-resistant hemagglutination of guinea pig eryth-

rocytes and were serologically K88 positive upon retesting, and vice versa, for K88-negative strains. Again, hydrophobic properties were independent of enterotoxin production.

Chromatography on Sepharose CL-4B. As an additional control to check for possible interactions of polysaccharide K antigens and/or O antigens with the polysaccharide gel, K88-positive and K88-negative strains of each O group in the Swedish and Dutch materials, including K88ab and K88ac variants where possible, were chromatographed on Sepharose CL-4B in the presence of buffered 4 M sodium chloride. None of the strains adsorbed visibly to this nonhydrophobic cross-linked agarose gel.

Identification of hydrophobic adsorptive properties with K88 antigen. K88-positive strains of each serogroup were grown on glucose nutrient agar at 37 and 18°C, and suspensions of these agar-grown bacteria were examined in parallel for K88 antigen serologically, for mannose-resistant hemagglutination and for adsorption to phenyl and octyl Sepharose. Cells grown at 18°C lacked K88 antigen and mannose-resistant hemagglutinating activity for guinea pig erythrocytes and did not adsorb to the hydrophobic gels, whereas bacteria grown at 37°C possessed all three properties. Thus, phenotypic expression of K88 antigen was necessary for hydrophobic surface characteristics.

Isaacson et al. (19) have described nonhemagglutinating pili (guinea pig eythrocytes) on porcine isolates of enteropathogenic *E. coli*. These appear to facilitate mucosal adherence and are morphologically similar but serologically distinct from mannose-sensitive common-type pili of porcine *E. coli* (19). However, the occurrence of such pili on K88-positive and K88-negative strains has not been thoroughly investigated. To determine whether or not such pili could be a contributing factor to hydrophobic interaction, Group V strains (Table 1), which lack K88 antigen, were examined by HIC. These were grown on both glucose nutrient agar and on heart infusion agar at 37°C (19). Neither strain 987 nor natural mutants derived from it which lacked the K antigen and/or nonhemagglutinating pili of the parent adsorbed to hydrophobic gels in the presence of 4 M sodium chloride.

Free-zone electrophoresis of K88-positive and K88-negative *E. coli*. Because of the differences in hydrophobic properties between K88-positive and K88-negative bacteria, free-zone electrophoresis was performed on serologically paired *E. coli* strains with and without K88 antigen (strains G1253 and D357). K88-negative bacteria had a higher migration rate than K88-positive bacteria toward the anode. The electrophoretic diagram for K88-positive bacteria showed two peaks, the minor one with an identical mobility to K88-negative bacteria, the major with lower mobility. The higher electrophoretic mobility of K88-negative bacteria indicates a greater negative surface charge density.

Effect of the ligand structure and salting-out ions on hydrophobic interaction. Three aryl and three alkyl chain-substituted derivatives of Sepharose were tested to determine the effect of ligand structure on the relative adsorptions of K88-positive and K88-negative bacteria. Figure 2 and Table 4 demonstrate that, in low molarity sodium phosphate buffer, 10 to 40% of the K88-positive bacteria adsorbed to the various hydrophobic gel ligands, compared with only 0 to 10% of the K88-negative bacteria. The adsorption of K88-positive bacteria was >99% at about 2 M sodium chloride for the ligands in

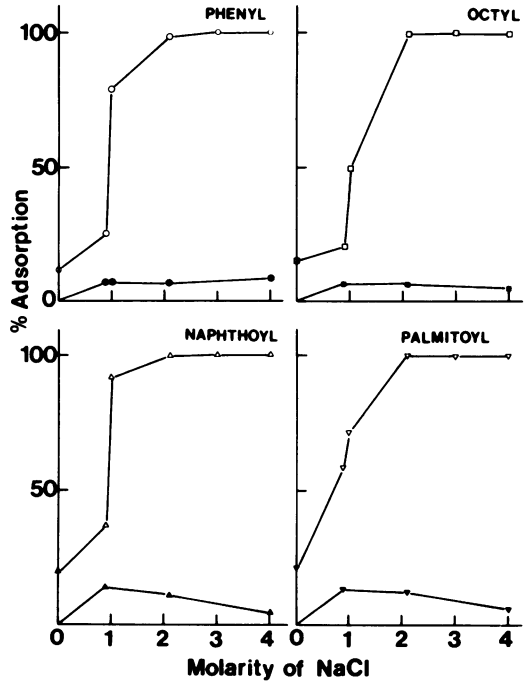

FIG. 2. *The relative adsorptions of K88-positive and K88-negative strains of E. coli to various hydrophobic gel ligands with increasing ionic strength. HIC was performed at 20°C. Percent adsorption is expressed as (100 − x)% of bacteria, where x is the percentage of applied bacteria in the column eluate. Symbols: △, ○, □, strain K-12(K88ab); ▲, ●, ■, strain K-12(K88⁻). The four different hydrophobic Sepharose gels investigated are indicated on the figure. Data points shown are mean values of duplicate determinations with the same batch of cells that was used with all four derivatives of Sepharose.*

TABLE 4. *Hydrophobic adsorption of paired K88⁺ and K88⁻ strains of E. coli to various hydrophobic ligands in the presence of 1 M ammonium sulfate*

Hydrophobic ligand[a]	Eluant	% of bacterial adsorption to gel[b]	
		K-12 (K88ab)	K-12 (K88⁻: K99⁻)
Phenyl	Buffer[c]	11.9	0
	1 M $(NH_4)_2SO_4$[d]	100	9.9
Octyl	Buffer	14.1	3.0
	1 M $(NH_4)_2SO_4$	100	4.5
Naphthoyl	Buffer	32.3	0.8
	1 M $(NH_4)_2SO_4$	100	41.1
Palmitoyl	Buffer	27.9	0
	1 M $(NH_4)_2SO_4$	100	0
Naphthyl	Buffer	38.1	0.7
	1 M $(NH_4)_2SO_4$	38.2	0
Dodecyl	Buffer	39.2	7.9
	1 M $(NH_4)_2SO_4$	90.7	7.4

[a] Naphthoyl gel beds were 10 mm long; all others were 30 mm long.

[b] Determined by measurements of A_{600} in a light path of 10 mm relative to viable counts.

[c] Sodium phosphate buffer (pH 6.8) was at 10 mM.

[d] Containing 10 mM sodium phosphate buffer (pH 6.8).

Fig. 2. With the degrees of substitution of the hydrophobic gels tested, adsorption to derivatized Sepharose with aryl ligands tended to be greater than to the derivatives with alkyl ligands in the presence of about 1 M sodium chloride. However, no systematic investigation of the effect of the degrees of substitution with these various ligands was made (36). The actual adsorption values with 0 to 2 M sodium chloride (in percent) varied considerably from experiment to experiment with different batches of cells of strain K-12 (K88ab), i.e., ±10 to 15% of the applied samples. This could be due in part to methodological errors such as variations in the light-scattering properties of bacteria in various molarities of sodium chloride (the performance of viable counts for each experiment was impracticable) and variations in the quantitative production of K88 antigen. However, the overall picture presented held true for comparative runs performed with different batches of cells.

Adsorption of K88-negative bacteria reached 5 to 20% at 1 M sodium chloride depending on the ligand, but this interaction reached a plateau value or decreased with increasing ionic strength (Fig. 2). Figure 2 also illustrates why it was easy to distinguish between K88-positive and K88-negative strains in the presence of 4 M sodium chloride when HIC was used as a screening procedure. The 5 to 10% of K88-negative cells which adsorbed was not readily visible. However, with 15 to 20% of K88-negative strains there appeared to be slight "adsorption" to the upper surface of the gel beds. Such adsorption, which occurred independently of salt concentration, was not interpreted to indicate hydrophobic interaction (16, 36).

Not only ionic strength but also the effectiveness of different salts in promoting hydrophobic interactions affect HIC (16, 17, 36). Because of the possible effects of high osmolality on the surface exposure of hydrophobic groups, ammonium sulfate, which is particularly effective in increasing hydrophobic interactions, was tried at lower molarity [0.15 M NaCl, 0.28 osmoles (osmol) per kg; 1 M $(NH_4)_2SO_4$, 1.92 osmol/kg, 2 M NaCl, 3.89 osmol/kg; 3 M NaCl, 6.98 osmol/kg; 4 M NaCl, 9.08 osmol/kg (51); the ionic strength of 1 M $(NH_4)_2SO_4$ corresponds to that of 3 M NaCl]. Clear-cut differences in adsorption were obtained for K88-positive and K88-negative bacteria with phenyl, octyl, and palmitoyl derivatives of Sepharose, but adsorption of K88-negative bacteria increased well above background with the naphthoyl ligand (Table 4). K88-positive bacteria did not adsorb well to naphthyl and dodecyl Sepharoses in the presence of 3 M sodium chloride, or to the former with 1 M ammonium sulfate. The reasons for the latter differences are unclear, but may be related to the degrees of substitution (36).

At concentrations of sodium chloride between 0 and 2 M, the adsorption patterns to phenyl and palmitoyl Sepharoses resembled visually that obtained in Fig. 1 with octyl Sepharose in the presence of 4 M sodium chloride. Distinct bands of adsorbed bacteria only appeared above 3 M sodium chloride. Compact adsorption zones were obtained with all gels except naphthyl Sepharose in the presence of 1 M ammonium sulfate.

A representative selection of the Dutch and Swedish clinical isolates were subsequently screened on palmitoyl, phenyl, and octyl Sepharoses in the presence of 1 M ammonium sulfate. The findings paralleled those described for 4 M sodium chloride experiments and for K-12 (K88ab) in 1 M ammonium sulfate.

DISCUSSION

This investigation presents a novel method for quick and convenient analysis of the hydrophobic surface properties of bacteria. In contrast to phase partition in a two-polymer system (25, 26, 45, 46), HIC requires simple apparatus and is

more amenable to use as a rapid screening procedure. Moreover, hydrophobic gels are readily available from different commercial sources. However, neutral gels such as those used herein are to be recommended for screening for hydrophobic characteristics to exclude electrostatic interactions.

With the phase partition systems, heat-killed radiolabeled bacteria have mainly been used. Although this may be permissible when studying the hydrophobicity of bacteria in relation to their O-group lipopolysaccharides, heat-killed and chemically modified bacteria may not have hydrophobic surface properties identical to those possessed by untreated bacteria, especially those with heat-labile adhesins such as K88 and K99 antigens, colonization factor, or other new colonization antigens (6, 7, 10, 20, 29; D. G. Evans, D. J. Evans, Jr., and T. R. Deetz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B8, p. 15; J. P. Arbuthnott and C. J. Smyth, *In* D. C. Ellwood, J. Melling, and P. Rutter (ed.), *Adhesion of Microorganisms to Surfaces,* in press). Indeed, external labeling of bacteria with ^{125}I appears to increase the hydrophobicity of these labeled bacteria according to their behavior in the palmitoyl—polyethylene glycol/dextran system compared to cells intrinsically labeled with [^{3}H]leucine (K.-E. Magnusson, University of Linköping, Sweden, personal communication).

In contrast, the present investigation has used freshly prepared suspensions of bacteria. The effect of high-salt concentrations on bacterial morphology and/or exposure of cell surface components imposes certain limitations on the interpretation of data when extrapolating to the naturally exposed surface under various physiological conditions. It must be emphasized that the performance of HIC in high-salt concentrations is designed to accentuate hydrophobicity. Such hydrophobic potentialities as detected under these conditions are probably present under certain physiological conditions (e.g., Fig. 2, palmitoyl Sepharose) as a component contributing to overall surface characteristics. Thus, the technique seems valid particularly for comparative analyses of the hydrophobic properties of strains such as the serologically paired strains with and without K88 antigen used in this investigation.

The reduced charge density on K88-positive bacteria observed by free-zone electrophoresis suggests that this pilus-like antigen neutralizes to some extent the negative charge of the surface of K88-negative *E. coli.* Similar differences in surface charge density have been reported by Heckels et al. (13), using isoelectric focusing to examine piliated and nonpiliated gonococci. The common type 1 pili (mannose sensitive) of *E. coli* have also been reported to reduce the surface charge density (3, 4). Moreover, like K88 antigen and gonococcal pili, these common-type pili contain a high proportion of apolar amino acids (4, 14, 20, 48) and thus might be thought potentially to confer hydrophobic characteristics on such piliate strains of *E. coli.* If common type 1 pili are present on the porcine isolates in this study under the growth conditions used, they would not appear to contribute to hydrophobicity, as it is unlikely that all K88-negative strains lack such pili. Other investigations on enterotoxigenic *E. coli* of human origin which exhibit mannose-sensitive hemagglutination of guinea pig erythrocytes suggest that common type 1 pili do not confer hydrophobic adsorptive properties on such bacteria (T. Wadström, C. J. Smyth, A. Faris, P. Jonsson, and J. H. Freer, submitted for publication). However, serological differences are known to occur between common type 1 pili (20) which probably reflect differences in structure and thus possibly hydrophobicity. Nonhemagglutinating pili of the type described by Isaacson et al. (19), however, do not confer hydrophobic adsorptive characteristics.

The findings herein strongly suggest that possession of certain types of adhesins by enteropathogenic *E. coli* might confer upon them hydrophobic characteristics not expressed on cells lacking such nonflagellar filaments. Our investigations have accordingly been extended to test this hypothesis. K99-positive and K99-negative strains from pigs, lambs, and calves and colonization factor-positive and -negative strains from cases of infantile diarrhea behave similarly to K88-positive and K88-negative strains on HIC (T. Wadström et al., submitted for publication). Moreover, hydrophobic interaction correlated with hemagglutinating properties and phenotypic expression of K99 and colonization factor at different incubation temperatures. Thus, HIC may prove to be a useful alternative for screening bacterial isolates for new colonization factors which might be missed by hemagglutination tests because of erythrocyte species specificity. However, run conditions may have to be modified for other bacterial systems.

Evidence to date suggests that the receptor on porcine intestinal brush border epithelium for K88 antigen is glycoprotein in nature (9, 19, 39). Indirect evidence based on inhibition of hemagglutination indicated that β-D-galactosyl residues in the heterosaccharide moieties of the membrane glycoproteins might be determinants of host tissue specificity. A lectin-like affinity between adhesin and cell membrane receptor or some sort of affinity reaction between complementary chemical groupings might be envisaged (27, 28; Arbuthnott and Smyth, in press).

Of what relevance is hydrophobic interaction

then? The lectin analogy is not as irrelevant as it might appear at first sight. Adjacent to the proposed carbohydrate-binding site of concanavalin A, there appears to be a hydrophobic region, and the binding site itself may comprise a prominent molecular cavity which is lined almost exclusively with hydrophobic groups (1, 24). Thus, this lectin possesses hydrophobic regions which may conceivably interact with other types of molecules on the cell surface, e.g., lipids. Indeed, five plant lectins, including concanavalin A, have recently been shown to bind specifically to liposomes containing membrane-associated glycolipids (2). Moreover, concanavalin A bound to liposomes without glycolipid (2) and induced fusion of pure dipalmitoylphosphatidylcholine liposomes (50). Thus, in the case of K88 antigen (and other bacterial adhesins) lectin-like binding dictating host tissue specificity may also involve hydrophobic interaction and allow such interaction between larger areas of the bacterial cell body and the host cell membrane, leading to the establishment of a noncovalent adhesive bond (Arbuthnott and Smyth, in press).

In summary, this report provides evidence for the suspected role of hydrophobic interaction in pili-mediated adhesion by enteropathogenic *E. coli* (20). It is suggested that reduction in the cell surface potential by the masking of the charge contribution of polysaccharide K antigens and lipopolysaccharide by certian pilus-like factors with hydrophobic characteristics probably promotes adhesion and that hydrophobic bonds are probably involved in the interaction of these adhesins with their target intestinal mucosal epithelia. Such adhesins thus provide a driving force for hydrophobic interaction with cell membranes.

ACKNOWLEDGMENTS

We express our sincere thanks to P. A. M. Guinée, C. Gyles, H. W. Moon, F. and I. Ørskov for supplying strains; to the Ørskovs for serotyping strains; to P. A. M. Guinée for enterotoxin data on Dutch strains; to K.-E. Magnusson for permission to cite unpublished observations and for useful discussion; to K. Elenbring, M. Glad, K. Krovacek, S. Mattsson, G. Sigstam, and K. Zimmerman for skillful technical assistance; and to S. Holdt and A.-M. Fogdegård for excellent secretarial service.

This study was supported by grants from the Swedish Medical Research Council to T.W. (16X-04723), the Swedish Council for Forestry and Agricultural Research to O.S. (40P-4576), and the Swedish Natural Science Research Council to S.H.

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