INFECTION AND IMMUNITY, Sept. 1996, p. 3694–3702 Vol. 64, No. 9
0019-9567/96/$04.00+0

Characterization of a Novel Hemagglutinin of Diarrhea-Associated *Escherichia coli* That Has Characteristics of Diffusely Adhering *E. coli* and Enteroaggregative *E. coli*

TATSUO YAMAMOTO,[1]* NORIKO WAKISAKA,[1] TAIJI NAKAE,[2] TOSHIKI KAMANO,[3] ORALAK SERICHANTALERGS,[4] AND PETER ECHEVERRIA[4]

Departments of Infectious Diseases and Tropical Medicine, Research Institute, International Medical Center of Japan,[1] and First Department of Surgery, School of Medicine, Juntendo University,[3] Tokyo, and Institute of Medical Sciences, School of Medicine, Tokai University, Isehara,[2] Japan, and Department of Bacteriology, Immunology, and Molecular Genetics, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand[4]

Received 14 November 1995/Returned for modification 12 December 1995/Accepted 21 June 1996

***Escherichia coli* 73-1 (serotype O73:H33) and 5-2 (serotype O89:H−) isolated from patients with diarrhea adhered to tissue culture cells (HeLa and HEp-2) as well as coverslips (plastic and glass) in a diffuse pattern. Adherence of strain 73-1 was mediated by a 110-kbp plasmid designated pEDA1 and correlated with D-mannose-resistant hemagglutinin (MRHA) detected with bovine, sheep, or human erythrocytes. The MRHA region was duplicated on pEDA1 and mediated the production of the 57-kDa outer membrane protein whose N-terminal amino acid sequence was hydrophobic. In accordance with MRHA and adherence, the 57-kDa outer membrane protein was observed best at 37°C and to a lesser extent at 25°C. In human intestine, adherence to mucus and colonic epithelium was obvious. No detectable pili were observed. The enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) gene, whose nucleotide sequence was 99.1% homologous to that of enteroaggregative *E. coli*, was present adjacent to the MRHA region on pEDA1. Strain 5-2 also exhibited MRHA activities and adherence and had sequences corresponding to those of the MRHA region and EAST1 gene. The data suggest that strain 73-1 (and strain 5-2), which has characteristics of both diffusely adhering *E. coli* and enteroaggregative *E. coli*, possesses a novel hemagglutinin associated with diffuse adherence.**

Although *Escherichia coli* is a major constituent of the normal flora of the human intestine, particular *E. coli* strains are associated with diarrhea. On the basis of distinct virulence properties and syndromes, diarrheagenic *E. coli* strains have been classified into five categories (1, 9, 19, 23): enterotoxigenic *E. coli* strains, enteropathogenic *E. coli* strains (EPEC), enteroinvasive *E. coli* strains, enterohemorrhagic *E. coli* strains, and enteroaggregative (previously termed enteroadherent-aggregative [23, 34]) *E. coli* strains (EAggEC). Among those, EPEC and EAggEC exhibit unique adherence patterns (localized adherence and aggregative adherence, respectively) in tissue culture cell assays using HEp-2 or HeLa cells (23, 24, 29).

In such tissue culture cell assays, a third type of adherence pattern (called diffuse adherence) has been demonstrated with *E. coli* isolated from patients with diarrhea (24, 29). Diffuse adherence was first reported with *E. coli* strains having EPEC serotypes (24, 29). Those strains carried adherence-mediating plasmids (2, 24) and produced no detectable hemagglutinins (HAs) (2, 24). The adherence factor of one such strain was shown to be a plasmid-encoded outer membrane protein of 100 kDa, called afimbrial adhesin involved in diffuse adherence I (AIDA-I) (3).

Another type of diffuse adherence was reported with diarrhea-associated *E. coli* strains (of non-EPEC serotypes) that were negative for the virulence factors of the five categories of diarrheagenic *E. coli*. Those strains have been called diffusely adherent *E. coli* (DAEC) (32) or diffuse-adhering *E. coli* (11) strains. In one such strain, the determinant for an adhesin (F1845 fimbriae) has been shown to be located on the chromosome (4). F1845 is a member of the Dr family of adhesins, which recognize the Dr(a) blood group antigen as a receptor. This family includes adhesins of uropathogenic *E. coli* such as afimbrial adhesins I and III and Dr hemagglutinin (26, 31). The F1845 DNA probe-positive DAEC adhere to tissue culture cells but not to glass (11, 34) and produce D-mannose-resistant HA (MRHA) detected with human erythrocytes (4). Volunteer studies using such F1845 DNA probe-positive DAEC have failed to demonstrate significant pathogenicity (32). Consequently, the association of diffuse adherence with diarrhea has remained unclear (8, 11, 19, 20). In other DAEC which did not hybridize with F1845 or AIDA DNA probes, the genes for diffuse adherence were located on conjugative drug resistance plasmids (13).

In previous experiments (38), we have analyzed bacterial adherence to HeLa cells by scanning electron microscopy and unambiguously demonstrated that there exist two distinct subtypes of DAEC: one adhering to HeLa cells but not to a plastic coverslip and being F1845 DNA probe positive, and another adhering to both HeLa cells and a plastic coverslip in a diffuse pattern and being F1845 DNA probe negative. The latter bacterial strains (73-1 and 5-2) had originally been reported to be EAggEC on the basis of their adherence patterns on HEp-2 cells observed under a light microscope (34). In this study, we investigated adherence of strains 73-1 and 5-2 to tissue culture cells (HeLa and HEp-2), coverslips (plastic and glass), and human intestines and characterized a novel HA associated with the adherence. We also determined the nucleotide sequence of the EAggEC heat-stable enterotoxin 1 (EAST1) gene, reported by Savarino et al. (27, 28), of strain 73-1.

* Corresponding author. Mailing address: Departments of Infectious Diseases and Tropical Medicine, Research Institute, International Medical Center of Japan, 1-21-2 Toyama, Shinjuku-ku, Tokyo, Japan. Fax: 81-3-3202-7364.

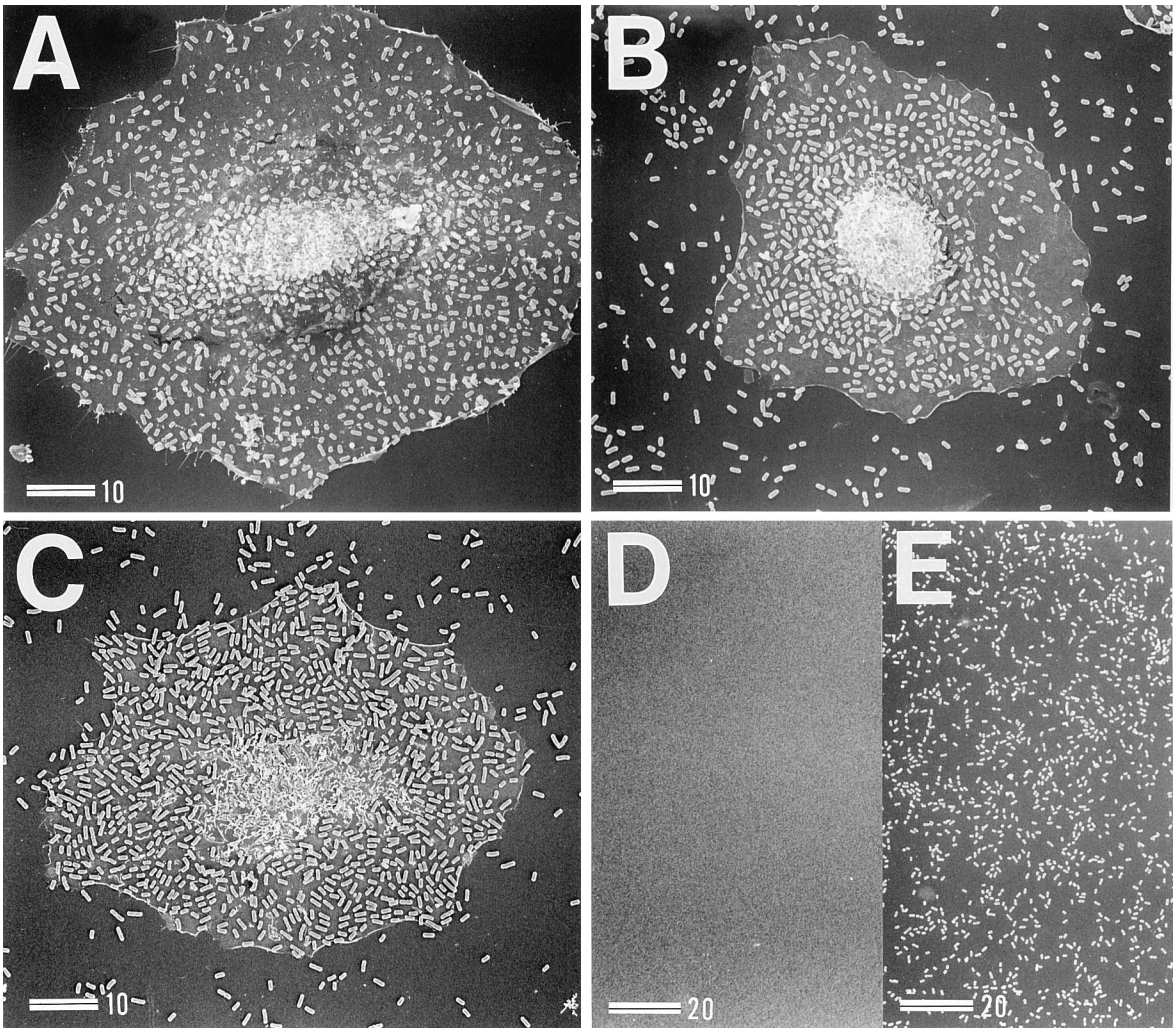

FIG. 1. Scanning electron micrographs showing adherence to HeLa cells and plastic coverslips of F1845 DNA probe-positive DAEC strain D1 (A and D), *E. coli* 73-1 (B and E), and *E. coli* 5-2 (C). (A to C) Adherence to HeLa cells on a plastic coverslip; (D and E) adherence to plastic coverslips (without tissue culture cells). Bars = 10 μm (A to C) and 20 μm (D and E).

MATERIALS AND METHODS

Bacterial strains. *E. coli* 73-1 (serotype O73:H33 [34, 38]) and 5-2 (serotype O89:H− [34]) were isolated from patients with diarrhea in Chile and kindly provided by James B. Kaper (University of Maryland School of Medicine, Baltimore). Strains 73-1 and 5-2 were negative (38) for colony hybridization with the EAggEC DNA probe (0.8-kbp *Pst*I-*Eco*RI fragment of pCVD432 which originated in the adherence-mediating plasmid of EAggEC) (1) or with the F1845 DNA probe (0.45-kbp *Pst*I fragment of pSLM852 which originated in the *daaC* gene on the chromosomal F1845 locus [4]); this probe is not specific for F1845 and may identify members of a Dr family of adhesins.

DAEC strain D1 (serotype O36:H4 [38]) was isolated from a patient with diarrhea in Thailand. This strain adhered to HeLa cells (in a diffuse pattern) but not to plastic coverslips and was positive with the F1845 DNA probe.

EAggEC strain TL100 (serotype O127a:H21 [37, 38]) was isolated from a patient with diarrhea in Thailand, adhered to both HeLa cells and plastic coverslips in an aggregative pattern, and was positive for the EAggEC DNA probe (1).

E. coli HB101 is a hybrid between *E. coli* K-12 and *E. coli* B and lacks restriction ability. *E. coli* 20S0 (K-12 derivative strain) carrying plasmid pTH10 was constructed previously (37); pTH10 is a self-transmissible plasmid (incompatibility group P) with temperature-sensitive replication and codes for resistance to ampicillin (as a result of the ampicillin resistance transposon Tn*1*), tetracycline, and kanamycin (12).

E. coli T19 (maltoporin $[LamB]^+$) and its $LamB^-$ mutant strain TNE001 were from collections in the Institute of Medical Sciences, Tokai University.

Media and bacterial growth. For bacterial growth, we used L broth (18) as a liquid medium. Incubations were conducted at 37°C for 14 to 20 h with agitation (unless otherwise noted). L (2%) agar, MacConkey agar (Eiken Chemical, Tokyo, Japan), and Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) were used as solid media. Incubations were carried out for 18 to 20 h at 37°C.

HA assay. Bacterial cells grown in L broth or on L agar were suspended in phosphate-buffered saline (PBS; pH 7.4) to a concentration of 600 Klett units (measured in a Klett-Summerson photoelectric colorimeter with a red filter; Klett Manufacturing, Long Island City, N.Y.). This concentration of DAEC strain 73-1 corresponded to 2.1×10^9 CFU/ml. HA titers were then determined by the 24-well plate method described previously (39).

Adherence test with tissue culture cells and coverslips. Adherence of *E. coli* strains to HeLa and HEp-2 cells was examined (unless otherwise noted) essentially by a method previously described (23, 29, 33) except that samples were finally examined with a scanning electron microscope (38) instead of a light microscope. HeLa or HEp-2 cells were grown on a plastic coverslip (diameter, 13.5 mm; Sumitomo Bakelite, Tokyo, Japan) at ~50% confluence; the plastic coverslips were those treated for hydrophilicity with negative charge at the surface. The cells on a plastic coverslip were then incubated in 1 ml of Eagle minimal essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5% fetal calf serum and 0.5% (wt/vol) D-mannose and containing 5 or 40 μl of bacterial cultures for 3 h at 37°C (38). Adherence of bacteria to plastic or glass coverslips (without cells) was also examined as described above.

In some experiments, bacterial adherence to plastic coverslips (with or without HeLa cells) was conducted in PBS for 10 min at 37°C as described previously (38).

Preparation of intestine specimens. Jejunum specimens were obtained from a 3-month-old infant with congenital biliary atresia at Juntendo Hospital. The mucosa of the intestine segment was isolated as described previously (39). The

TABLE 1. MRHA production of F1845 DNA probe-negative and -positive DAEC and EAggEC

E. coli strain	Medium	Time (h) of incubation at 37°C	MRHA activity[a] against erythrocytes of the indicated origin					
			Bovine	Sheep	Human	Guinea pig	Rabbit	Horse
F1845-negative diffusely adherent								
73-1	L broth	40	16 (4)	16 (4)	2 (<1)	2 (<1)	2 (<1)	2 (<1)
		20	8 (1)	8 (1)	1 (<1)	1 (<1)	1 (<1)	1 (<1)
	L agar	20	1	<1	<1	<1	<1	<1
5-2	L broth	40	4 (1)	4 (1)	<1 (<1)	<1 (<1)	<1 (<1)	<1 (<1)
		20	2 (<1)	2 (<1)	<1 (<1)	<1 (<1)	<1 (<1)	<1 (<1)
	L agar	20	<1	<1	<1	<1	<1	<1
D1 (F1845-positive diffusely adherent)	L broth	40	<1 (<1)	<1 (<1)	16 (8)	<1 (<1)	<1 (<1)	<1 (<1)
		20	<1 (<1)	<1 (<1)	16 (8)	<1 (<1)	<1 (<1)	<1 (<1)
	L agar	20	<1	<1	16	<1	<1	<1
TL100 (enteroaggregative)	L broth	40	64 (32)	16 (16)	64 (32)	32 (32)	<1 (<1)	32 (16)
		20	32 (32)	16 (16)	32 (16)	32 (16)	<1 (<1)	32 (16)
	L agar	20	<1	<1	<1	<1	<1	<1

[a] Bacteria were grown in L broth with agitation or on L agar for the indicated times at 37°C. Data indicate the highest dilution (fold) yielding positive results by the 24-well plate method. A concentration of undiluted bacterial samples, corresponding to an HA titer of 1, was 600 Klett units in a colorimeter. D-Mannose was added to 0.5% (wt/vol). Values in parentheses are titers for bacteria grown in L broth without agitation.

mucosa was also fixed with 10% (vol/vol) Formalin and used for adherence as described previously (39). When adherence to mucus was examined, mucus blobs that were directly associated with goblet cells were analyzed.

Colon specimens were obtained from a 2-month-old infant with congenital biliary atresia at Juntendo Hospital. The mucosal side of the intestine was prepared and fixed with Formalin (37).

Intestine adherence test. Bacterial cells grown in L broth (for 20 h) were suspended in PBS to a concentration of 600 Klett units. D-Mannose was added at a concentration of 1% (wt/vol). Pieces of intestine specimens were immersed in 1.5 ml of the bacterial suspension and then incubated for 30 min at 28°C. The intestine samples with bound bacteria were washed in PBS and fixed with glutaraldehyde and osmium tetroxide. Fixed samples were analyzed by scanning electron microscopy (39).

Susceptibility testing. Susceptibility testing of bacterial strains was done by the agar dilution method with Mueller-Hinton agar, using the standard procedure (14). When susceptibility to sulfamethoxazole or trimethoprim was tested, Mueller-Hinton agar supplemented with 7.5% (vol/vol) defibrinated horse blood (frozen and thawed) was used instead of Mueller-Hinton agar alone. The MIC was determined as described previously (14).

Plasmid analysis and transformation with plasmid DNA. Plasmids in *E. coli* strains were analyzed (or isolated) essentially by a published method (15) as described previously (38). Plasmids in *E. coli* strains were also analyzed by another method (5). Transformation of *E. coli* HB101 with plasmid DNA was done as described previously (36).

Tn*1* labeling and insertion mutagenesis. Labeling of plasmid with Tn*1* and Tn*1* insertion mutagenesis were done by using plasmid pTH10 (Tn*1* donor) essentially as described previously (12, 37).

DNA cloning and sequencing. Cloning of the MRHA-associated region and the EAST1 gene into pBR322 (specifying resistance to ampicillin and tetracycline) or pACYC184 (specifying resistance to chloramphenicol and tetracycline) was done as described previously (40). DNA size was determined by using 8- to 48-kb DNA size standards (Bio-Rad Laboratories, Hercules, Calif.), a 1-kb DNA ladder (Life Technologies, Gaithersburg, Md.), and *Hae*III-digested ϕX174 replicative-form DNA fragments (Life Technologies) as molecular size standards. DNA sequences were determined by using a *Taq* Dyedeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and 373A DNA sequencer (Applied Biosystems) according to the manual.

DNA-DNA hybridization. DNA fragments were purified from agarose gel and labeled with digoxigenin by using a DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Southern hybridization was done as described previously (10), using a nylon membrane (Life Technologies). DNA hybrids on the membrane were treated with alkaline phosphatase-conjugated antidigoxigenin antibody and then with 3-(2′-spirodamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane (substrate), using a digoxigenin luminescence detection kit (Boehringer Mannheim Biochemicals). The resultant chemiluminescence was detected by exposing the membrane to X-ray film. Colony hybridization (10) was also done as described above.

Outer membrane protein analysis. The outer membrane fraction of *E. coli* HB101 with or without plasmids was prepared as described previously (30). Bacteria were grown in L broth for 16 to 18 h at various temperatures. The bacterial cells were then passed through a French pressure cell (1,100 kg/cm^2), and the outer membrane fraction was separated from the inner membrane fraction by centrifugation through a discontinuous sucrose gradient (0.77, 1.44, and 2.02 M sucrose in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4]). The outer membrane fraction of strain 73-1 (and *E. coli* HB101 with or without plasmids in some experiments) was prepared by a different method (21). In this experiment, bacteria were grown in L broth for 4 to 5 h, the inner membrane was solubilized with *N*-lauroylsarcosinate (final concentration, 1%), and the insoluble fraction of the outer membrane was obtained. Proteins associated with the outer membrane were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (17). Protein bands in the SDS-polyacrylamide gel (10 and 13%) were stained with Coomassie blue. When amino acid sequences (at the N termini) of the proteins were determined, protein bands in the SDS-polyacrylamide gel (10%) were transferred to a polyvinylidene difluoride membrane (pore size, 0.45 μm; Millipore) by electroblotting, and then sequencing was performed on an Applied Biosystems 477A pulse-liquid protein sequencer equipped with a 120A phenythiohydantoin-derived amino acid analyzer.

Transmission electron microscopy. Bacterial cells grown in L broth were

TABLE 2. MRHA levels and abilities to adhere to HeLa cells and plastic coverslips of *E. coli* 73-1 and HB101 carrying pEDA1-P12 grown at various temperatures

Strain	Temp (°C)	MRHA titer[a]	Adherence to[b]:	
			HeLa cells	Plastic coverslips
73-1	25	<1	8.7 ± 7.4	0.6 ± 0.9
	30	4	94.6 ± 44.9	28.5 ± 27.3
	37	16	96.1 ± 56.4	54.8 ± 19.5
	42	1	20.0 ± 17.1	6.0 ± 4.3
HB101(pEDA1-P12)	25	2	10.8 ± 7.8	5.1 ± 4.6
	30	32	107.7 ± 63.8	42.8 ± 24.2
	37	64[c]	258.9 ± 95.3	140.5 ± 24.8
	42	8	108.6 ± 70.0	62.8 ± 32.0
HB101	25	<1	0.3 ± 0.4	0.3 ± 0.4
	30	<1	0.2 ± 0.3	0.1 ± 0.3
	37	<1[d]	0.3 ± 0.3	0.2 ± 0.3
	42	<1	0.3 ± 0.3	0.2 ± 0.3

[a] Bacteria were grown in L broth for 20 h at the indicated temperatures with agitation. MRHA activities were examined with bovine erythrocytes. A bacterial concentration corresponding to an HA titer of 1 equals 600 Klett units. D-Mannose was added to 0.5% (wt/vol).

[b] The assay of adherence of bacteria (grown as described above and suspended to 600 Klett units in PBS) to HeLa cells or plastic coverslips was conducted for 10 min at 37°C. D-Mannose was added to 0.5% (wt/vol). Data for HeLa cells indicate numbers of bacterial cells adherent to a HeLa cell (means ± standard deviations for 30 determinations). Data for plastic coverslips indicate numbers of bacterial cells observed in a scanning electron microscopic field (37 by 50 μm) at a magnification of ×2,500.

[c] MRHA activities against other erythrocytes were 32 (sheep erythrocytes), 4 (human erythrocytes), 4 (guinea pig erythrocytes), 4 (rabbit erythrocytes), and 4 (horse erythrocytes).

[d] MRHA activities against sheep, human, guinea pig, rabbit, or horse erythrocytes were <1.

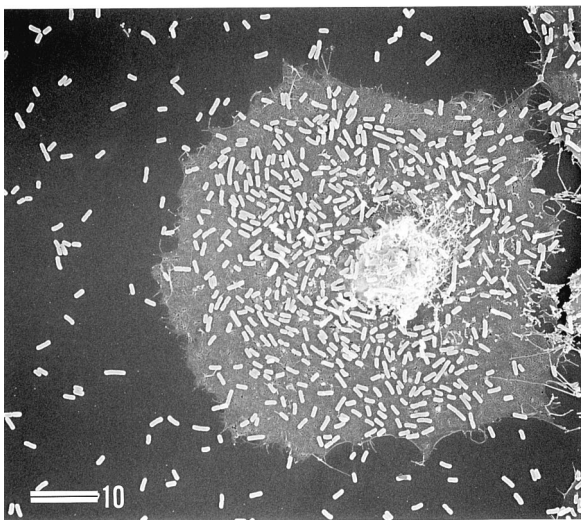

FIG. 2. Scanning electron micrograph showing adherence to HeLa cells on a plastic coverslip by *E. coli* HB101 carrying pBRH85. Bar = 10 μm.

suspended in water, and one drop of the suspension was applied to each of several collodion-coated grid screens. The adherent bacteria were negatively stained as previously described (37).

RESULTS

Adherence to tissue culture cells and coverslips. DAEC strain D1, which was positive for the F1845 DNA probe, adhered to HeLa or HEp-2 cells in a diffuse pattern (Fig. 1A) but not to plastic or glass coverslips (Fig. 1D). In electron microscopic study, EAggEC strain TL100 occurred in a pattern aggregating in chains or forming random aggregates on tissue culture cells as well as in areas free of tissue culture cells (data not shown) (38). In marked contrast, strains 73-1 and 5-2 adhered to HeLa or HEp-2 cells (Fig. 1B and C) as well as plastic or glass coverslips (Fig. 1E) in a diffuse pattern, although the adherence patterns were slightly more aggregative in the HEp-2 cell assay than in the HeLa cell assay. The adherence levels of strains 73-1 and 5-2 for tissue culture cells were comparable to or slightly greater than those for plastic or glass coverslips. Adherence levels to plastic coverslips diminished to 10.4% (for strain 73-1) and 4.1% (for strain 5-2) when plastic coverslips not treated for hydrophilicity were used.

MRHA production. When strain 73-1 was grown in L broth for 20 h at 37°C with agitation, marked MRHA activity was detected (Table 1). The MRHA activity was detected best with bovine or sheep erythrocytes and was lower against human, guinea pig, rabbit, and horse erythrocytes (Table 1). MRHA activity increased by ~2-fold with a longer incubation period (40 h) and decreased by 2- to 8-fold when cells were incubated in L broth without agitation or grown on agar plates. Moreover, higher levels of MRHA were detected at 30 or 37°C than at 25 or 42°C (Table 2).

Strain 5-2 exhibited similar MRHA activities (detected best with bovine or sheep erythrocytes) but at a lower level than did strain 73-1 (Table 1).

In contrast, DAEC strain D1 exhibited distinct MRHA activity (detected only with human erythrocytes) when grown in L broth as well as on L agar at 37°C (Table 1). MRHA activity of EAggEC strain TL100 was further distinct and detected with bovine, human, guinea pig, horse, and sheep erythrocytes (Table 1); this MRHA was detected when TL100 was grown in L broth at 37°C but not when TL100 was grown on L agar.

Large plasmids of strain 73-1. Strain 73-1 had two large plasmids with molecular sizes of 117 and 110 kbp. The 117-kbp plasmid coded for resistance to streptomycin, sulfamethoxazole, and trimethoprim; MICs of the drugs for strain 73-1 and *E. coli* K-12 strains carrying the 117-kbp plasmid were all ≥200 μg/ml. Since introduction of pTH10 into strain 73-1 resulted in loss of the 117-kbp plasmid, the 117-kbp plasmid belonged to incompatibility group P.

The 110-kbp plasmid (designated pEDA1) of strain 73-1 was labeled with ampicillin resistance transposon Tn*1* (originated in pTH10), and the labeled plasmids were introduced into *E. coli* HB101 by transformation. The transformants adhered to both HeLa (or HEp-2) cells and plastic (or glass) coverslips in a diffuse pattern. One such plasmid, pEDA1::Tn*1*, was designated pEDA1-P12. The transformants exhibited temperature-dependent MRHA production and adherence, as shown in Table 2.

By culturing a 73-1 derivative strain (that possessed pTH10 and lacked the 117-kbp plasmid) in L broth at 42°C, a strain carrying only pEDA1 was obtained. This clone was used as a source of pEDA1 DNA.

Cloning of the MRHA region. DNA of pEDA1-P12 was digested with *Hin*dIII or *Sal*I, the fragments were inserted into pBR322, and MRHA$^+$ transformants of *E. coli* HB101 were obtained. MRHA titers against bovine erythrocytes of those transformants were 16 to 32, and comparable to that of strain 73-1. These MRHA$^+$ transformants adhered to HeLa or HEp-2 cells and plastic or glass coverslips in a diffuse pattern, as shown in Fig. 2.

The MRHA$^+$ recombinant plasmids, which were obtained by *Hin*dIII digestion, consisted of a 19.3- or 13.5-kbp fragment of pEDA1-P12 (corresponding to the H1 or H3 fragment, respectively, of pEDA1) and a pBR322 fragment (Fig. 3, lanes 3 and 4). The recombinant plasmids possessing the 19.3- and 13.5-kbp fragments were designated pBRH82 and pBRH85, respectively. The MRHA$^+$ recombinant plasmids, which were obtained by *Sal*I digestion, consisted of a 13.8-kbp fragment of pEDA1-P12 (which was also present in pEDA1)

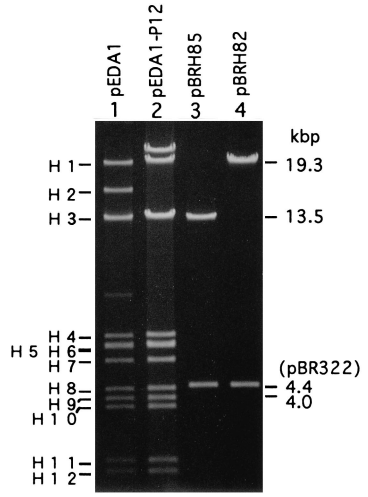

FIG. 3. *Hin*dIII digestion patterns of pEDA1 and related plasmids. DNA fragments were analyzed by electrophoresis in a 0.3% agarose gel and stained with ethidium bromide. Plasmids: pEDA1, adherence-mediating plasmid of strain 73-1; pEDA1-P12, MRHA$^+$ Tn*1* insert of pEDA1; pBRH85 and pBRH82, MRHA$^+$ recombinant plasmids of pBR322 possessing a *Hin*dIII fragment of pEDA1-P12. Fragment sizes (in kilobase pairs) of pEDA1 were as follows: H1, 19.3; H2, 15.6; H3, 13.5; H4, 6.5; H5, 6.1; H6, 6.0; H7, 5.4; H8, 4.3; H9, 4.0; H10, 3.7; H11, 2.4; H12, 2.2; H13, 1.3; H14, 1.1; H15, 0.97; H16, 0.70; and H17, 0.54.

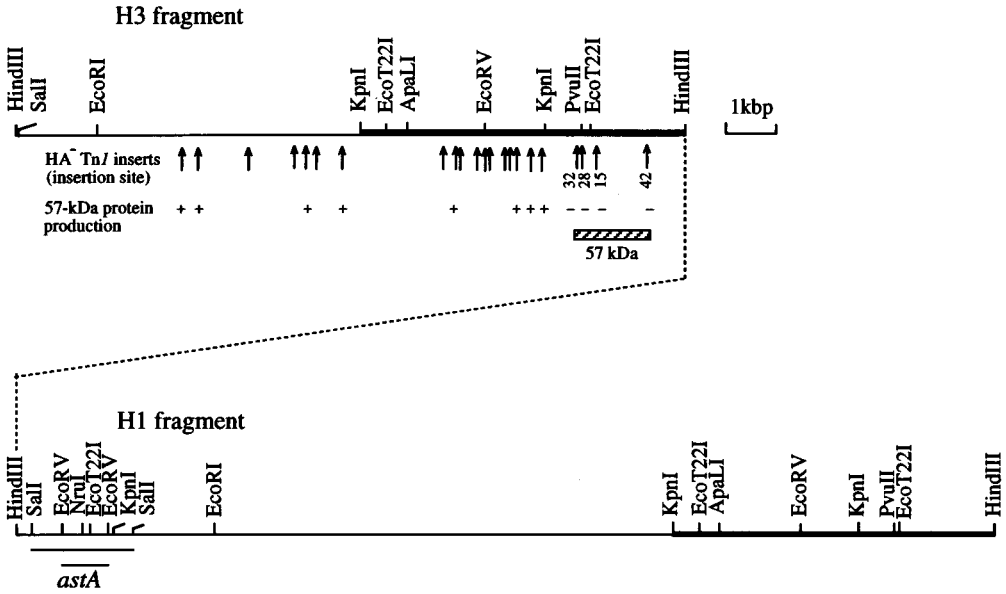

FIG. 4. Locations of the repeated MRHA-associated regions and EAST1 gene in the H3-H1 region of pEDA1-P12. The *Hin*dIII site at the right side of the H3 fragment corresponds to the *Hin*dIII site at the left side of the H1 fragment. The H3 fragment was inserted into pACYC184, and the resultant MRHA$^+$ recombinant plasmid (pACH8) was subjected to Tn*1* mutagenesis analysis. The Tn*1* insertion sites (within the H3 fragment) of the MRHA$^-$ inserts were determined and are indicated by arrows. *E. coli* strains carrying those MRHA$^-$ Tn*1* inserts of pACH8 displayed no adherence to tissue culture cells. The Tn*1* inserts that produced the 57-kDa outer membrane protein are marked +; the Tn*1* inserts that failed to produce the 57-kDa outer membrane protein (pACH8-32, pACH8-28, pACH8-15, and pACH8-42) are marked −. The thick lines (6.4 kbp long) in the H3 and H1 fragments represent the directly repeated regions with identical restriction maps. The EAST1 gene (*astA*) was cloned into pBR322 as a 2.0-kbp *Sal*I fragment or a 0.62-kbp *Eco*RV fragment (indicated by a bar).

and a pBR322 fragment; the recombinant plasmid was designated pBRS72.

The map of the H3 (13.5 kbp)-H1 (19.3 kbp) fragment region (32.8 kbp long) of pEDA1-P12 was constructed (Fig. 4). The H3 and H1 fragments were contiguous, because the MRHA-associated, 13.8-kbp *Sal*I fragment (of pBRS72) was present over the H3 fragment (Fig. 4) and the 0.3-kbp *Hin*dIII-*Sal*I fragment at the right side of the 13.8-kbp *Sal*I fragment was identical to the *Hin*dIII-*Sal*I fragment at the left side of the H1 fragment in terms of size and nucleotide sequence (data not shown).

Tn*1* mutagenesis analysis of the MRHA$^+$ H3 fragment. The H3 fragment of pBRH85 was inserted into pACYC184, and the resultant MRHA$^+$ recombinant plasmid, pACH8, was transferred to *E. coli* HB101 carrying pTH10 (Tn*1* donor) to obtain MRHA$^-$ Tn*1* inserts. Determination of Tn*1* insertion sites revealed that the MRHA-associated region occupied the bulk of the H3 fragment (Fig. 4). All of the HA$^-$ Tn*1* inserts showed no adherence to HeLa cells. The 6.4-kbp *Kpn*I-*Hin*dIII region was directly repeated in the H3 and H1 fragments.

The 1.9-kbp *Eco*T22I-*Hin*dIII fragment present at the right side of the H3 fragment (Fig. 4) was purified, labeled with digoxigenin, and used as a probe. This hybridization experiment showed that a sequence homologous to that of the 1.9-kbp *Eco*T22I-*Hin*dIII fragment was present in the H1 fragment and also in the 113-kbp plasmid (designated pEDA2) of strain 5-2.

Outer membrane protein analysis. Strain 73-1 and related strains were grown at 37°C, and proteins associated with the outer membrane were analyzed by SDS-PAGE (Fig. 5). Comparison of three samples, from strain 73-1 (Fig. 5A, lane 1), *E. coli* HB101 carrying MRHA$^+$ plasmid pEDA1-P12 (Fig. 5A, lane 2), and *E. coli* HB101 (Fig. 5A, lane 3), revealed that pEDA1 encoded two outer membrane proteins of 57 kDa and 30 kDa.

E. coli HB101 carrying an MRHA$^+$ recombinant plasmid (pBRH82, pBRH85 [or pACH8], or pBRS72) had only a 57-kDa outer membrane protein (Fig. 5B, lane 2). *E. coli* HB101 carrying an HA$^-$ Tn*1* insert of pACH8 (pACH8-32, pACH8-28, pACH8-15, or pACH8-42) failed to produce this 57-kDa protein (Fig. 5B, lane 3), indicating that the 57-kDa protein region is located at the right side of the H3 fragment (Fig. 4).

The 57-kDa protein bands of *E. coli* HB101 carrying pEDA1-P12, pBRH82, pBRH85, or pBRS72 (Fig. 5B, lanes 1 and 2) were extracted, and the amino-terminal amino acid sequence was determined. The sequences obtained with those 57-kDa proteins were identical to each other and hydrophobic (Fig. 5D).

Next, strain 73-1 and *E. coli* HB101 carrying pEDA1-P12 were grown at various temperatures, and then outer membrane proteins were analyzed. At 25°C (a temperature at which levels of MRHA and adherence were extremely low [Table 2]), a 57-kDa protein band disappeared and a 46-kDa protein band appeared (Fig. 5C, lane 1); the appearance of the 46-kDa protein band was due to the overproduction of LamB protein (7) in those strains, as evidenced by SDS-PAGE analysis with LamB protein and the amino-terminal amino acid sequence analysis of the 46-kDa protein (Fig. 5D). At 42°C, the bacteria still had some MRHA, displayed adherence, and had the 57-kDa protein to some extent.

Bacterial cells possessing MRHA activity (strain 73-1, *E. coli* HB101 carrying pEDA1-P12, pBRH82, pBRH85, or pBRS72, and strain 5-2) had no detectable pili when tested by transmission electron microscopy.

Adherence to human intestinal mucosa. The washed, control mucosa of child jejunum had blobs of mucus which were directly associated with goblet cells (Fig. 6A). Strain 73-1 adhered to the mucus (associated with goblet cells) at an extremely high level (Fig. 6B). In contrast, the adherence lev-

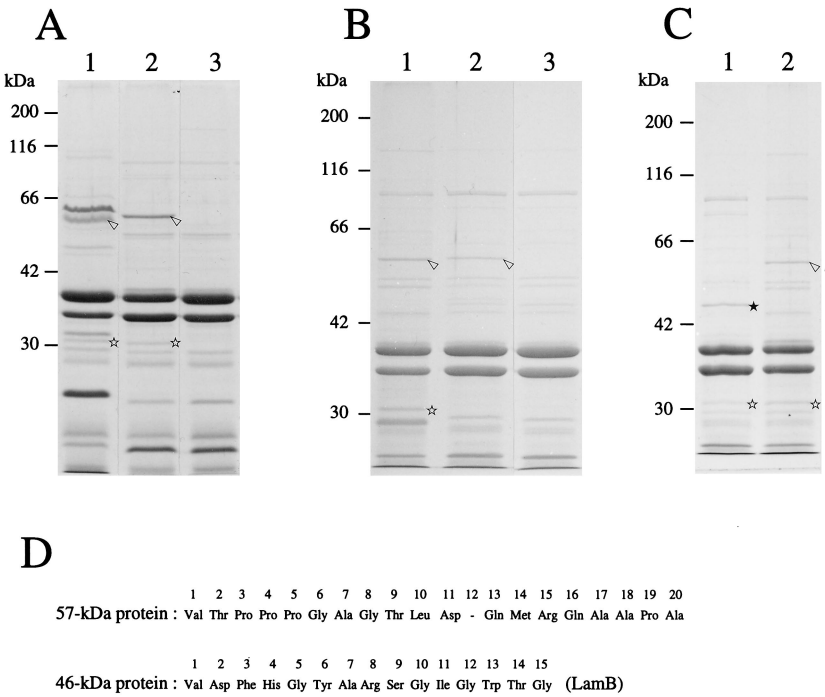

FIG. 5. Outer membrane protein analysis of *E. coli* 73-1 and related strains. Outer membrane proteins were analyzed by electrophoresis in SDS–10% polyacrylamide gels. Triangles, filled asterisks, and open asterisks, respectively, indicate 57-, 46-, and 30-kDa proteins. (A) Lane 1, strain 73-1; lane 2, *E. coli* HB101 carrying an MRHA$^+$ Tn*1* insert of pEDA1 (pEDA1-P12); lane 3, *E. coli* HB101. (B) Lane 1, *E. coli* HB101 carrying pEDA1-P12; lane 2, *E. coli* HB101 carrying pACH8 (MRHA$^+$ pACYC184 derivative possessing the 13.5-kbp *Hin*dIII fragment of pEDA1-P12); lane 3, *E. coli* HB101 carrying pACH8-28 (MRHA$^-$ Tn*1* insert of pACH8). In panels A and B, bacteria were grown at 37°C. (C) Lane 1, *E. coli* HB101 carrying pEDA1-P12 grown at 25°C; lane 2, *E. coli* HB101 carrying pEDA1-P12 grown at 37°C. (D) Amino-terminal amino acid sequences of the 57- and 46-kDa outer membrane proteins.

el of strain 73-1 to the epithelium (absorptive cells) was very low.

Adherence to the mucus was also demonstrated with Formalin-fixed intestine specimens. Strain 73-1 and *E. coli* HB101 carrying an MRHA$^+$ plasmid (pEDA1-P12, pBRH85, pBRH82, or pACH8) adhered well (Fig. 7C). In contrast, *E. coli* HB101 carrying an MRHA$^-$ plasmid (pACH8-32, pACH8-28, pACH8-15, or pACH8-42) displayed no adherence. Strain 5-2 also markedly adhered to the mucus (data not shown).

When Formalin-fixed colonic mucosa (Fig. 7A) was incubated with bacteria, strain 73-1 and *E. coli* HB101 carrying an MRHA$^+$ plasmid (pEDA1-P12, pBRH85, pBRH82, or pACH8) displayed marked adherence even to the epithelium (Fig. 7B and C). In contrast, *E. coli* HB101 carrying an MRHA$^-$ plasmid (pACH8-32, pACH8-28, pACH8-15, or pACH8-42) showed no adherence. Strain 5-2 also adhered well to Formalin-fixed colonic mucosa (data not shown).

Location and nucleotide sequence of the EAST1 gene on pEDA1. The EAST1 gene (*astA*) was located at the left side of the H1 fragment (adjacent to the MRHA region in the H3 fragment) (Fig. 4). The determined nucleotide sequence of the EAST1 gene differed from that of EAggEC strain 17-2 (28) by only one base at codon position 21 (ACA→GCA), resulting in a deduced amino acid change (Thr→Ala). The EAST1 gene was also present in the 113-kbp plasmid (pEDA2) of strain 5-2.

DISCUSSION

This study clearly demonstrated that adherence in a diffuse pattern of strain 73-1 is mediated by a 110-kbp plasmid (pEDA1). In addition, the data obtained in this study strongly indicated that the adherence factor of strain 73-1 is a plasmid-encoded, 57-kDa outer membrane protein with an MRHA activity. The production of the 57-kDa outer membrane protein was strictly regulated by growth temperature, and the production was best observed at 37°C. This finding, together with the fact that the 57-kDa outer membrane protein region was duplicated on the plasmid, suggests that the production of the 57-kDa outer membrane protein is important for virulence of strain 73-1.

The amino-terminal amino acid sequence of the 57-kDa protein contained a proline-rich Thr_2-Pro-Pro-Pro-Gly_6 region. This sequence was present in *E. coli* penicillin-binding protein 1A (an inner membrane protein) (6). Plastic coverslips used in this study were coated with hydrophilic residues such as COOH or OH. Since adherence of strain 73-1 to plastic coverslips drastically decreased when uncoated plastic coverslips were used, the 57-kDa protein may interact with hydrophilic residues on the surface.

Strain 73-1 (and strain 5-2) had originally been reported to be EAggEC on the basis of its adherence patterns on HEp-2 cells (34). Indeed, the two strains had properties similar to those of EAggEC: (i) strains 73-1 and 5-2 adhered to plastic (or glass) coverslips similarly to EAggEC, and the adherence patterns in HEp-2 cell assays tended to be slightly aggregative; (ii) strains 73-1 and 5-2 adhered well to mucus in human small intestines and human colonic mucosa, similarly to EAggEC (16, 37); (iii) MRHA was detected in L broth better than on L agar, similarly to EAggEC; and (iv) strains 73-1 and 5-2 had sequences of the EAST1 gene (*astA* [28]) originally found in an EAggEC strain, 17-2.

However, there exist marked differences between strains 73-1 and 5-2 and EAggEC. (i) Strains 73-1 and 5-2 adhered

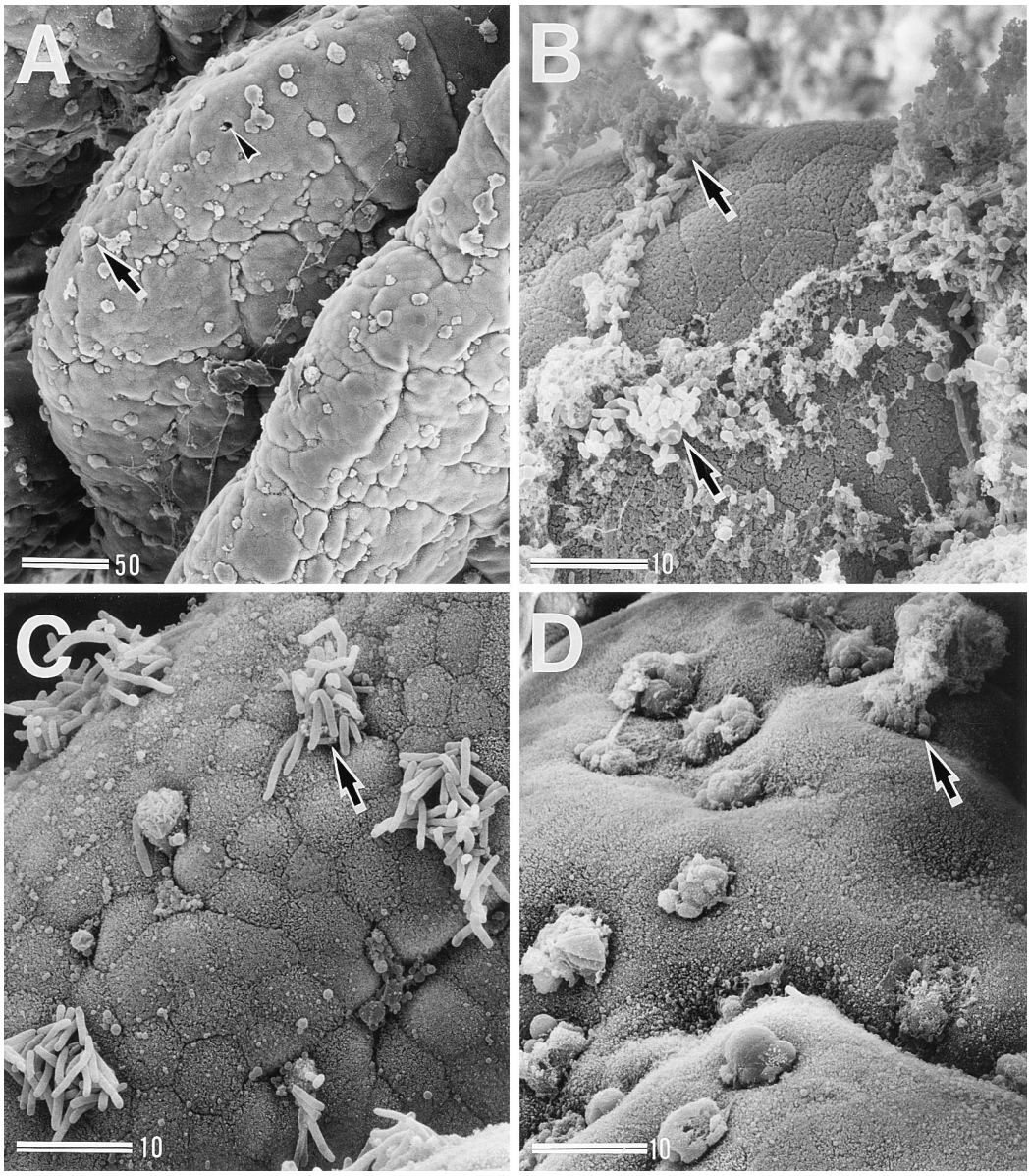

FIG. 6. Scanning electron micrographs showing adherence to human jejunal mucosa by *E. coli* 73-1, HB101 carrying the MRHA$^+$ plasmid pBRH85, and HB101 carrying the MRHA$^-$ plasmid pACH8-28. (A) Washed, control mucosa. Arrowhead and arrow, respectively, indicate a goblet cell (observed as a hole when viewed from the lumen side) and a blob of mucus associated with a goblet cell. (B) Adherence of strain 73-1 to native mucus associated with a goblet cell (arrows indicate adherent bacteria). (C) Adherence of *E. coli* HB101 carrying the MRHA$^+$ plasmid pBRH85 to mucus of the Formalin-fixed specimen (arrow indicates adherent bacteria) (HB101 carrying the MRHA$^+$ plasmid pACH8 also adhered to mucus). (D) Lack of adherence of *E. coli* HB101 carrying the MRHA$^-$ plasmid pACH8-28 to mucus of the Formalin-fixed specimen (arrow indicates mucus not bound with bacteria). Bars = 10 μm (B to D) and 50 μm (A).

to HeLa cells and plastic (or glass) coverslips in a typical pattern of diffuse adherence, in contrast to EAggEC (this study and references 23, 34, and 38). (ii) An adherence factor of strain 73-1 is the 57-kDa outer membrane protein, while an adherence factor of EAggEC is pili (aggregative adherence fimbriae I [22, 25]). No DNA sequences homologous to the MRHA-associated region of pEDA1 were present in EAgg- EC (data not shown). (iii) MRHA titers of strains 73-1 and 5-2 were high against bovine erythrocytes and low against human erythrocytes, while MRHA titers of EAggEC were high against both bovine and human erythrocytes (Table 1) (37).

We have recently shown that the EAST1 gene is not unique to EAggEC and is distributed among ETEC in association with the adherence factor type (35). The EAST1 gene sequence of strain 73-1 was identical to those of ETEC. Therefore, the presence of the EAST1 gene sequence is now not a strict marker of EAggEC.

Strain 73-1 (possessing the 57-kDa outer membrane protein) is distinct from F1845$^+$ DAEC (possessing pili and exhibiting MRHA detected best with human erythrocytes [4]) or from AIDA-I$^+$ DAEC (possessing the 100-kDa outer membrane protein [2, 3, 24]).

Studies of pathogenicity are required with a new subtype of DAEC (strains 73-1 and 5-2) that has a novel adhesin and the EAST1 gene.

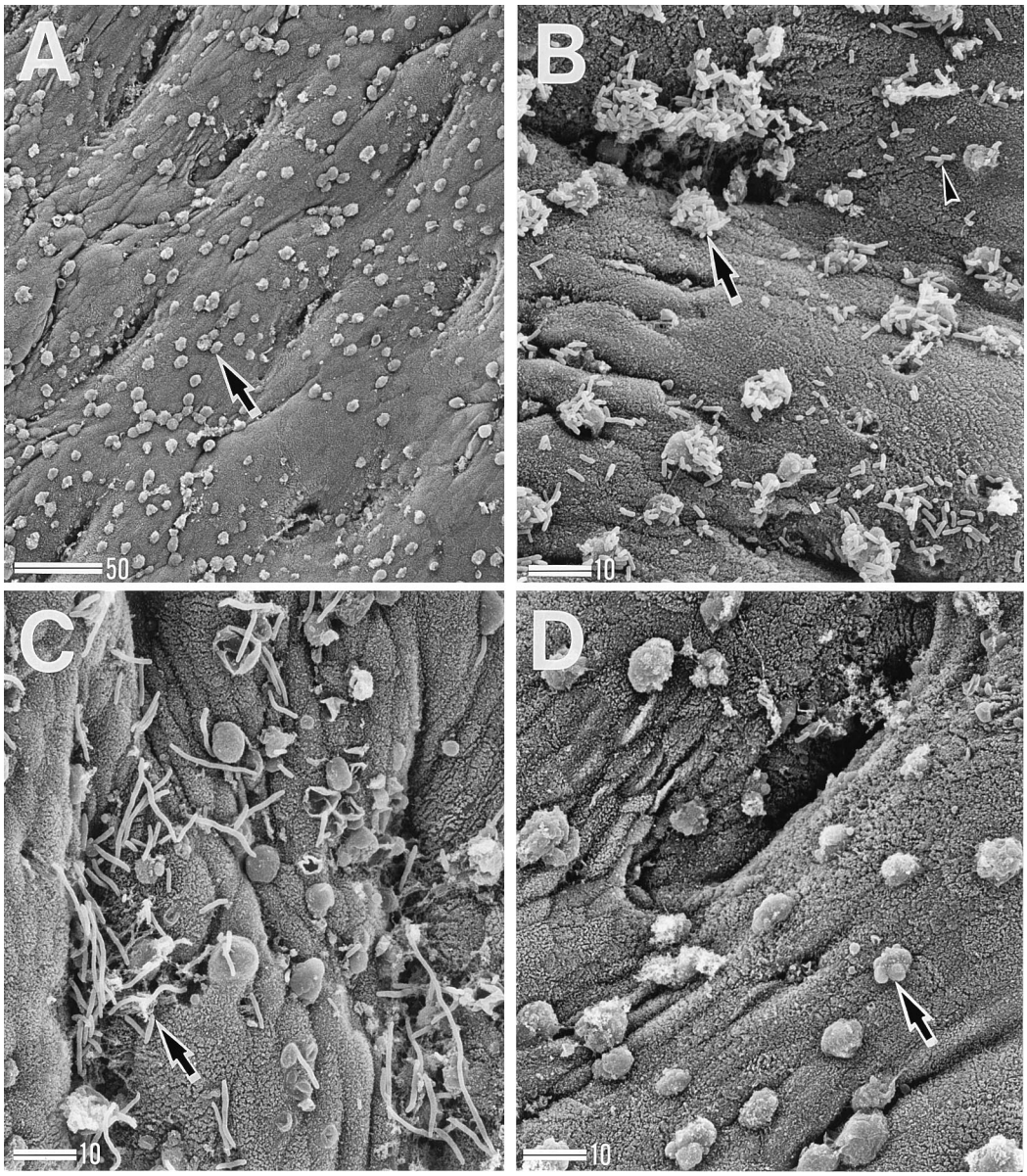

FIG. 7. Scanning electron micrographs showing adherence to Formalin-fixed child colonic mucosa by *E. coli* 73-1, HB101 carrying the MRHA$^+$ plasmid pBRH85, and HB101 carrying the MRHA$^-$ plasmid pACH8-28. (A) Washed, control mucosa (arrow indicates mucus); (B) adherence of strain 73-1 (arrow and arrowhead, respectively, indicate bacteria bound to mucus and the epithelium); (C) adherence of *E. coli* HB101 carrying the MRHA$^+$ plasmid pBRH85 (arrow indicates adherent bacteria); (D) lack of adherence of *E. coli* HB101 carrying the MRHA$^-$ plasmid pACH8-28 (arrow indicates mucus not bound with bacteria). Bars = 10 μm (B to D) and 50 μm (A).

ACKNOWLEDGMENTS

We thank James B. Kaper for bacterial strains, Eisaku Yosihara and Hiroshi Yoneyama for kind suggestions, and Yoshifumi Takeda for continuous interest and encouragement.

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan and a grant from the Department of Health and Welfare of Japan.

REFERENCES

1. **Baudy, B., S. J. Savarino, P. Vial, J. B. Kaper, and M. M. Levine.** 1990. A sensitive and specific DNA probe to identify enteroaggregative *Escherichia coli*: a recently discovered diarrheal pathogen. J. Infect. Dis. **161:**1249–1251.
2. **Benz, I., and M. A. Schmidt.** 1989. Cloning and expression of an adhesin (AIDA-I) involved in diffuse adherence of enteropathogenic *Escherichia coli*. Infect. Immun. **57:**1506–1511.
3. **Benz, I., and M. A. Schmidt.** 1992. Isolation and serologic characterization of AIDA-I, the adhesin mediating the diffuse adherence phenotype of the diarrhea-associated *Escherichia coli* strain 2787 (O126:H27). Infect. Immun. **60:**13–18.
4. **Bilge, S. S., C. R. Clausen, W. Lau, and S. L. Moseley.** 1989. Molecular characterization of a fimbrial adhesin, F1845, mediating diffuse adherence of diarrhea-associated *Escherichia coli* to HEp-2 cells. J. Bacteriol. **171:**4281–4289.
5. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
6. **Broome-Smith, J. K., A. Edelman, S. Yousif, and B. G. Spratt.** 1985. The nucleotide sequences of the *ponA* and *ponB* genes encoding penicillin-binding proteins 1A and 1B of *Escherichia coli* K12. Eur. J. Biochem. **147:**437–446.
7. **Clément, J. M., and M. Hofnung.** 1981. Gene sequence of the λ receptor, an outer membrane protein of *E. coli* K12. Cell **27:**507–514.
8. **Cravioto, A., A. Tello, A. Navarro, J. Ruiz, H. Villafan, F. Uribe, and C. Eslava.** 1991. Association of *Escherichia coli* HEp-2 adherence patterns with

type and duration of diarrhoea. Lancet **337:**262–264.
9. **Echeverria, P., S. T. Savarino, and T. Yamamoto.** 1993. *Escherichia coli* diarrhoea. Baillière's Clin. Gastroenterol. **7:**243–262.
10. **Echeverria, P., J. Seriwatana, O. Sethabutr, and A. Chatkaeomorakot.** 1990. Detection of diarrheogenic *Escherichia coli* using nucleotide probes, p. 95–141. *In* A. J. L. Macario and E. C. de Macario (ed.), Gene probes for bacteria. Academic Press, San Diego, Calif.
11. **Girón, J. A., T. Jones, F. Millán-Velasco, E. Castro-Munoz, L. Zárate, J. Fry, G. Frankel, S. L. Moseley, B. Baudry, J. B. Kaper, G. K. Schoolnik, and L. W. Riley.** 1991. Diffuse-adhering *Escherichia coli* (DAEC) as a putative cause of diarrhea in Mayan children in Mexico. J. Infect. Dis. **163:**507–513.
12. **Hirayama, S., M. Tsuda, and T. Iino.** 1980. High frequency mobilization of the chromosome of *Escherichia coli* by a mutant of plasmid RP4 temperature-sensitive for maintenance. Mol. Gen. Genet. **180:**47–56.
13. **Jallat, C., V. Livrelli, A. Darfeuille-Michaud, C. Rich, and B. Joly.** 1993. *Escherichia coli* strains involved in diarrhea in France: high prevalence and heterogeneity of diffusely adhering strains. J. Clin. Microbiol. **31:**2031–2037.
14. **Japan Society of Chemotherapy.** 1981. Committee report. Chemotherapy (Tokyo) **29:**76–79.
15. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
16. **Knutton, S., R. K. Shaw, M. K. Bhan, H. R. Smith, M. M. McConnell, T. Cheasty, P. H. Williams, and T. J. Baldwin.** 1992. Ability of enteroaggregative *Escherichia coli* strains to adhere in vitro to human intestinal mucosa. Infect. Immun. **60:**2083–2091.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1:**190–206.
19. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. **155:**377–389.
20. **Levine, M. M., V. Prado, R. Robins-Browne, J. Lior, J. B. Kaper, S. L. Moseley, K. Gicquelais, J. P. Nataro, P. Vial, and B. Tall.** 1988. Use of DNA probes and HEp-2 cell adherence assay to detect diarrheagenic *Escherichia coli*. J. Infect. Dis. **158:**224–228.
21. **Masuda, N., E. Sakagawa, and S. Ohya.** 1995. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**645–649.
22. **Nataro, J. P., Y. Deng, D. R. Maneval, A. L. German, W. C. Martin, and M. M. Levine.** 1992. Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infect. Immun. **60:**2297–2304.
23. **Nataro, J. P., J. B. Kaper, R. Robins-Browne, V. Prado, P. Vial, and M. M. Levine.** 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. Pediatr. Infect. Dis. J. **6:**829–831.
24. **Nataro, J. P., I. C. A. Scaletsky, J. B. Kaper, M. M. Levine, and L. R. Trabulsi.** 1985. Plasmid-mediated factors conferring diffuse and localized adherence of enteropathogenic *Escherichia coli.* Infect. Immun. **48:**378–383.
25. **Nataro, J. P., D. Yikang, J. A. Giron, S. J. Savarino, M. H. Kothary, and R. Hall.** 1993. Aggregative adherence fimbria I expression in enteroaggregative *Escherichia coli* requires two unlinked plasmid regions. Infect. Immun. **61:** 1126–1131.
26. **Nowicki, B., A. Labigne, S. Moseley, R. Hull, S. Hull, and J. Moulds.** 1990. The Dr hemagglutinin, afimbrial adhesins AFA-I and AFA-III, and F1845 fimbriae of uropathogenic and diarrhea-associated *Escherichia coli* belong to a family of hemagglutinins with Dr receptor recognition. Infect. Immun. **58:**279–281.
27. **Savarino, S. J., A. Fasano, D. C. Robertson, and M. M. Levine.** 1991. Enteroaggregative *Escherichia coli* elaborate a heat-stable enterotoxin demonstrable in an in vitro rabbit intestinal model. J. Clin. Invest. **87:**1450–1455.
28. **Savarino, S. J., A. Fasano, J. Watson, B. M. Martin, M. M. Levine, S. Guandalini, and P. Guerry.** 1993. Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of *E. coli* heat-stable toxin. Proc. Natl. Acad. Sci. USA **90:**3093–3097.
29. **Scaletsky, I. C. A., M. L. M. Silva, and L. R. Trabulsi.** 1984. Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLa cells. Infect. Immun. **45:**534–536.
30. **Smit, J., Y. Kamio, and H. Nikaido.** 1975. Outer membrane of *Salmonella typhimurium*: chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. J. Bacteriol. **124:**942–958.
31. **Swanson, T. N., S. S. Bilge, B. Nowicki, and S. Moseley.** 1991. Molecular structure of the Dr adhesin: nucleotide sequence and mapping of receptor-binding domain by use of fusion constructs. Infect. Immun. **59:**261–268.
32. **Tacket, C. O., S. L. Moseley, B. Kay, G. Losonsky, and M. M. Levine.** 1990. Challenge studies in volunteers using *Escherichia coli* strains with diffuse adherence to HEp-2 cells. J. Infect. Dis. **162:**550–552.
33. **Vial, P. A., J. J. Mathewson, H. L. DuPont, L. Guers, and M. M. Levine.** 1990. Comparison of two assay methods for patterns of adherence to HEp-2 cells of *Escherichia coli* from patients with diarrhea. J. Clin. Microbiol. **28:**882–885.
34. **Vial, P. A., R. Robins-Browne, H. Lior, V. Prado, J. B. Kaper, J. P. Nataro, D. Maneval, A. Elsayed, and M. M. Levine.** 1988. Characterization of enteroadherent-aggregative *Escherichia coli*, a putative agent of diarrheal disease. J. Infect. Dis. **158:**70–79.
35. **Yamamoto, T., and P. Echeverria.** 1996. Detection of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene sequences in enterotoxigenic *E. coli* strains pathogenic for humans. Infect. Immun. **64:**1441–1445.
36. **Yamamoto, T., P. Echeverria, and T. Yokota.** 1992. Drug resistance and adherence to human intestines of enteroaggregative *Escherichia coli*. J. Infect. Dis. **165:**744–749.
37. **Yamamoto, T., S. Endo, T. Yokota, and P. Echeverria.** 1991. Characteristics of adherence of enteroaggregative *Escherichia coli* to human and animal mucosa. Infect. Immun. **59:**3722–3739.
38. **Yamamoto, T., Y. Koyama, M. Matsumoto, E. Sonoda, S. Nakayama, M. Uchimura, W. Paveenkittiporn, K. Tamura, T. Yokota, and P. Echeverria.** 1992. Localized, aggregative, and diffuse adherence to HeLa cells, plastic, and human small intestines by *Escherichia coli* isolated from patients with diarrhea. J. Infect. Dis. **166:**1295–1310.
39. **Yamamoto, T., and T. Yokota.** 1988. Electron microscopic study of *Vibrio cholerae* O1 adherence to the mucus coat and villus surface in the human small intestine. Infect. Immun. **56:**2753–2759.
40. **Yamamoto, T., T. Yokota, and A. Kaji.** 1981. Molecular organization of heat-labile enterotoxin genes originating in *Escherichia coli* of human origin and construction of heat-labile toxoid-producing strains. J. Bacteriol. **148:** 983–987.

Editor: B. I. Eisenstein