Aggregative Adherence Fimbria II, a Second Fimbrial Antigen Mediating Aggregative Adherence in Enteroaggregative *Escherichia coli*

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Enteroaggregative *Escherichia coli* (EAEC) has been implicated as an agent of pediatric diarrhea in the developing world. We have shown previously that EAEC adheres to HEp-2 cells by virtue of a plasmid-encoded fimbrial adhesin designated aggregative adherence fimbria I (AAF/I), the genes for which have been cloned and sequenced. However, not all EAEC strains express AAF/I. Using *TnphoA* mutagenesis, we have characterized a novel fimbria (designated AAF/II) which mediates HEp-2 adherence of the human-pathogenic strain 042. AAF/II is 5 nm in diameter and does not bind AAF/I antiserum, as determined by immunogold transmission electron microscopy. *TnphoA* identified a gene (designated *aafA*) which bears significant homology to *aggA*, the fimbrial subunit of AAF/I (25% identity and 47% similarity at the amino acid level). When hyperexpressed and purified by polyhistidine tagging, the AafA protein assembled into 5-nm-diameter filaments which bound anti-AAF/II antiserum. The cloned *aafA* gene complemented a mutation in the *aggA* gene to confer fimbrial expression from the AAF/I gene cluster, manifesting phenotypes characteristic of AAF/II but not AAF/I. The *aafA* mutant did not adhere to human intestinal tissue in culture, suggesting a role for AAF/II in intestinal colonization. By using DNA probes for AAF/I and AAF/II derived from fimbrial biosynthesis genes, we show that AAF/I and AAF/II are each found in only a minority of EAEC strains, suggesting that still more EAEC adhesins exist. Our data suggest that AAF adhesins represent a new family of fimbrial adhesins which mediate aggregative adherence in EAEC.

Enteroaggregative *Escherichia coli* (EAEC) is a cause of diarrhea in the developing world (4, 5, 8, 44) and is defined by its characteristic aggregative adherence (AA) to HEp-2 cells in culture (31). The pathogenesis of EAEC diarrhea is not completely defined; however, two prominent pathogenic features have been described: (i) formation of a thick mucus gel on the intestinal mucosa (12, 41) and (ii) mucosal damage, apparently via the elaboration of a cytotoxin(s) (12, 30). One or more enterotoxins may also play a role in EAEC diarrhea (2, 37).

It has been shown that the AA phenotype in EAEC strains is associated with the presence of a plasmid 60 to 65 MDa in size (the AA plasmid) (29, 32, 43) and that AA in EAEC strain 17-2 is mediated by a plasmid-encoded fimbrial structure 2 to 3 nm in diameter (29, 34). This fimbria, designated aggregative adherence fimbria I (AAF/I), is encoded by two unlinked regions of plasmid DNA separated by 9 kb (34). AAF/I region 1 encodes a cluster of four genes involved in fimbrial biogenesis (38); region 2 encodes an AraC homolog (designated AggR) which is required for fimbrial expression (28). However, the lack of hybridization of the AAF/I biogenesis genes with other EAEC strains has suggested that many strains do not express this fimbrial antigen (33), despite the apparent relatedness of AA plasmids (43).

Here, we report that the AA phenotype of a human-virulent EAEC strain, 042, is encoded on the AA plasmid but that AA is due to the presence of a fimbrial structure that is genetically,

phenotypically, and morphologically distinct from AAF/I expressed by strain 17-2. The fimbrial adhesin of strain 042, herein named AAF/II, mediates adherence to human colonic mucosa in tissue explants and is thus a candidate for a human intestinal colonization factor.

MATERIALS AND METHODS

Strains and plasmids. Bacterial strains used in this study are listed in Table 1. Strain 042 was isolated from a child with diarrhea in the course of an epidemiologic study in Lima, Peru, in 1983 (27); this strain has been shown to cause diarrhea in adult volunteers (33). EAEC strains used in retrospective colony blot analysis were from the collection of the Center for Vaccine Development and were isolated during epidemiologic studies in various sites throughout the developing world. All strains were stored at -70°C in Trypticase soy broth with 15% glycerol. *E. coli* HB101 or DH5 α was used as a recipient strain for genetic manipulations (1, 6). Strains were routinely passaged on Luria-Bertani broth (L broth) or agar with the following antibiotics where appropriate: ampicillin (200 $\mu\text{g/ml}$), kanamycin (50 $\mu\text{g/ml}$), streptomycin (100 $\mu\text{g/ml}$), tetracycline (15 $\mu\text{g/ml}$), and chloramphenicol (20 $\mu\text{g/ml}$).

Molecular cloning and nucleotide sequence analysis. All genetic manipulations were performed by standard methods (1). Plasmid DNA was extracted by using a Plasmid Midi kit (Qiagen Inc., Chatsworth, Calif.). Purification of DNA fragments and extraction from agarose gel slices were performed with GeneClean (Bio 101, La Jolla, Calif.). Plasmid DNA was introduced into *E. coli* HB101 by transformation of competent cells (Gibco/BRL, Gaithersburg, Md.) according to the method of Hanahan (11).

DNA sequence determination was performed by using a model 373A automated sequencer (Applied Biosystems, Foster City, Calif.) via dye terminator cycle sequencing with *Taq* polymerase (Perkin-Elmer Corp., Norwalk, Conn.) according to the manufacturers' instructions. Sequencing was performed by primer walking on both strands in the Biopolymer Laboratory, Department of Microbiology and Immunology, University of Maryland School of Medicine. DNA sequences were analyzed with Genepro sequence analysis software (version 5.00; Riverside Scientific, Bainbridge Island, Wash.) and the Wisconsin Genetics Computer Group sequence analysis package available through the Center of Marine Biotechnology, University of Maryland. The predicted amino

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Description ^a	Reference or origin
Strains		
042	Wild-type EAEC strain; expresses AAF/II	27
17-2	Wild-type EAEC strain; expresses AAF/I	43
HS	Wild-type normal-flora strain	21
HB101	K-12/B hybrid	6
042::phoA 3.4.14	Wild-type EAEC strain with AA-inactivating <i>TnphoA</i> insertion (<i>aafA</i> mutant)	This study
Plasmids		
pBluescript II SK	High-copy-number cloning vector (Ap)	Stratagene
pACYC177	Low-copy-number cloning vector (Ap Km)	New England Biolabs
pRK415	Low-copy-number cloning vector (Tc)	15
pAA2	65-MDa adherence plasmid of EAEC strain 042	32
pAA2::phoA 3.4.14	pAA2 with AA-inactivating <i>TnphoA</i> insertion in <i>aafA</i> gene	This study
pJPN31	AAF/I-encoding cosmid clone derived from strain 17-2 (Tc)	29
pJPN32	pJPN31 with AAF/I-inactivating <i>TnphoA</i> insertion	29
pJC2	1.7-kb <i>EcoRI</i> fragment of pAA2 encoding <i>aafA</i> , cloned into pBluescript SK II (Ap)	This study
pJC3	0.8-kb <i>cat</i> cassette cloned into <i>SmaI</i> site adjacent to insert of pJC2 (Ap Cm)	This study
pJPN39	6.8-kb <i>ClaI</i> fragment from strain 17-2 containing region 1 of AAF/I cloned into <i>ClaI</i> site of vector pACYC177 (Ap)	This study
pJPN39Ω4	Omega interposon cloned into <i>HincII</i> site of pJPN39, inactivating the <i>aggA</i> gene (Ap Sm)	This study
pQE70	Polyhistidine expression vector (Ap)	Qiagen
pJPN45	3.0-kb <i>KpnI-XbaI</i> fragment of encoding <i>aggR</i> cloned into pRK415 (Tc)	34

^a Abbreviations: Ap, ampicillin resistance; Tc, tetracycline resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance.

acid sequence of each open reading frame (ORF) was compared with proteins listed in EMBL/GenBank by using the Genetics Computer Group TF/ASTA program and the BLAST algorithm (National Center for Biotechnology Information).

Colony blot hybridization was performed by standard methods (1, 10); 25 ng of probe fragment was labeled by random primer extension (Prime-it kit; Stratagene Cloning Systems, La Jolla, Calif.) with 50 μ Ci of [³²P]dATP (Amersham Life Science Products, Arlington Heights, Ill.). Southern hybridization was performed by standard methods (1). The *aggA* probe was derived by PCR with the following primers: 5' TTAGTCTCTATCTAGGG and 3' AAATTAATTCCG GCATGG.

Protein expression and purification. The *aafA* gene product was expressed by cloning a 550-bp fragment generated by PCR (primers, 5' ACATGCATGCAAA AAATCAGAATGTTTGT and 3' CGGGATCCATTGTGTCACAAGCTCA GC) into the *Bam*HI and *Sph*I sites of the expression vector pQE70 (Qiagen). The protein was thereby synthesized as a fusion with six histidines linked to the carboxy terminus. Protein expression was achieved by incubating an L-broth culture of the construct at 37°C with shaking until an optical density at 650 nm of 0.6 to 0.7 was reached; at that time, isopropyl- β -D-thiogalactopyranoside (IPTG) (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 0.2 mM. The fusion protein was purified from a whole-cell French press lysate by passage through a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen) and pH-dependent elution, both procedures according to the manufacturer's instructions. The column eluate was dialyzed overnight against 1 mM MgCl₂ at 4°C.

Amino-terminal amino acid sequencing was performed after proteins were separated by polyacrylamide gel electrophoresis on gradient gels of 8 to 14% with the Phast system (Pharmacia, Piscataway, N.J.). The proteins were transblotted onto polyvinylidene membranes, were excised, and were sequenced by automated Edman degradation essentially as described previously (25), using a model 477A protein sequencer (Applied Biosystems) equipped with a model 120A analyzer.

Complementation analysis. The ability of the *aafA* gene to complement a null mutation in the *aggA* gene (the fimbrial subunit of AAF/I) was tested as follows. A 250-bp *HincII* fragment internal to the *aggA* gene was removed from AAF/I region 1 clone pJPN39 (Table 1), and the 1.7-kb omega interposon with *SmaI* ends (36) was cloned into the remaining *HincII* site, thereby generating pJPN39Ω4. Unlike pJPN39, pJPN39Ω4 is unable to confer AAF/I expression even in the presence of the transcriptional activator AggR, provided by the chimeric plasmid pJPN45 (Table 1). A *trans*-complementation experiment in which the *aafA* gene was provided by pJC3, consisting of the 1.7-kb *EcoRI* fragment expressing *aafA* cloned into the corresponding site of pBluescript SK II, was then performed. To provide the necessary antibiotic selection, the 0.8-kb *cat* cassette expressing chloramphenicol resistance (23) was cloned into the pBluescript *SmaI* site upstream of the *EcoRI* fragment. All three plasmids (pJPN39Ω4, pJPN45, and pJC3) were introduced by transformation into strain DH5 α . Selection and maintenance of the three plasmids required the presence of ampicillin, chloramphenicol, streptomycin, and tetracycline.

Transposon *TnphoA* mutagenesis. Insertion mutagenesis of strain 042 was performed by using transposon *TnphoA* (24) as previously described (29, 40). Strain *E. coli* SM10 λ pir(pRT733) was cross-streaked against strain 042 (streptomycin resistant) on L-agar plates, and the plates were incubated at 37°C overnight. The growth from each plate was then harvested and plated on L agar containing streptomycin and kanamycin and 50 μ g of 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co.). Blue colonies were selected for analysis. This mutagenesis strategy yielded 480 alkaline phosphatase-expressing fusions as a result of 62 separate matings. Fusions were numbered so that the products of different matings could be identified. Screening of mutants in the HEP-2 assay yielded 55 insertion mutants which were altered in their adherence phenotype.

Preparation of antiserum. HB101(pAA2) was grown for 18 h in L broth at 37°C without shaking, was then washed in phosphate-buffered saline (PBS), and was injected intravenously into 1.5-kg New Zealand White rabbits as described previously (34). Two weeks after completion of the immunization course, the rabbits were bled and the serum was separated. Antiserum was absorbed against the AAF/II mutant HB101(pAA2::phoA 3.4.14) until the antiserum failed to bind the organism upon immunogold electron microscopy (EM). AAF/I antiserum production (by a similar technique) was described previously (34).

HEP-2 adherence assay. The HEP-2 adherence assay was performed as described previously (7, 42), using a 3-h incubation. Coverslips were examined under oil immersion light microscopy for the characteristic stacked-brick pattern of adherence to HEP-2 cells and to the glass coverslip. Assays were scored as AA positive if they yielded at least one aggregate (of more than four bacteria) per high-power field. Each assay was performed in triplicate. A nonadherent mutant was defined as one with less than one aggregate per high-power field.

For adherence inhibition studies, 20 μ l of bacterial culture and 50 μ l of antiserum (diluted in PBS) were added simultaneously to tissue culture wells containing HEP-2 cells, and the adherence assay was performed as described above. After 3 h of incubation, bacterial aggregates (defined as more than four autoagglutinating bacteria) were counted in four fields under $\times 400$ power, and data were expressed as mean numbers of aggregates per field.

EM. Transmission EM of negatively stained specimens was performed by standard methods (22) on a JEOL JEM 1200 EX II transmission electron microscope. Anti-AAF/I and anti-AAF/II antisera were used at a 1/100 dilution in immunogold EM as described previously (29).

HA. Hemagglutination (HA) assays were performed as described by Yamamoto et al. (45). Anticoagulated bovine blood and sheep blood were obtained from PML Microbiologicals (Tualatin, Oreg.). Human, rat, and rabbit blood specimens were collected by venipuncture. Erythrocytes were washed twice in PBS and were resuspended to a final concentration of 3% (vol/vol) with 1% (wt/vol) mannose. Bacteria for the HA assay were grown overnight in L broth at 37°C without shaking. Bacteria were harvested and resuspended to a density of 10⁸ CFU/ml in PBS. A 100- μ l volume of bacterial suspension was mixed with 100 μ l of erythrocyte suspension in 24-well Falcon polystyrene cell culture dishes (Becton Dickinson, Lincoln Park, N.J.); after being gently mixed, the reaction mixture was incubated for 20 min at room temperature. Positive HA results were

FIG. 1. Transmission EM of *E. coli* HB101 expressing AAF/I or AAF/II. (a) HB101 transformed with pAA2, the adherence plasmid of strain 042. Transformants acquire AA as well as the presence of rigid, 5-nm-diameter AAF/II fimbriae. Bar, 500 nm. (b) HB101(pJPN31), which expresses AAF/I. AAF/I filaments are 2 to 3 nm in diameter and typically form bundles. Bar, 200 nm.

FIG. 2. Immunogold EM with anti-AAF/I antiserum. (a) AAF/I-positive control strain 17-2. (b) AAF/II-producing strain 042. Bars, 200 nm.

recorded as visible without magnification (++) or as visible only by inverted microscopy at $\times 100$ magnification (+).

Inhibition of HA was performed by combining 100 μ l of the bacterial inoculum and 100 μ l of the antiserum (serially diluted 1:2 in PBS) and then incubating the mixture at room temperature for 30 min. A 100- μ l sample of this suspension was added to an equal volume of the erythrocyte suspension, and the HA reaction was performed as described above.

Polystyrene adherence. Polystyrene adherence was assayed by growing the test bacteria overnight at 37°C in a polystyrene 24-well dish (Falcon Industries) containing 1 ml of L broth. After overnight incubation, the broth was discarded and the dish was stained for 1 min with 10% Giemsa stain. The Giemsa stain was then discarded, and the plate was washed three times with tap water. The assay was considered positive if a blue film was observed on the surface of the culture dish after the staining. The presence of adherent bacteria was confirmed by inverted microscopy.

IVOC. EAEC strain 042 and afimbrial mutant 042::phoA 3.4.14 were examined for adhesion to human jejunal and colonic mucosae. Prior to in vitro organ culture (IVOC) studies, strains were subcultured into brain heart infusion broth and incubated at 37°C without agitation for 18 h. Endoscopically and histologically normal mucosal samples from the jejunum were obtained from three pediatric patients (ages 9, 36, and 98 months), and normal mucosal samples from the transverse colon were obtained from another three patients (ages 150, 174, and 180 months) all undergoing routine endoscopic investigation to rule out inflammatory bowel disease. The IVOC assay was essentially as described by Knutton et al. (16, 17). The intestinal mucosa was incubated with the *E. coli* strain for 8 h at 37°C in 95% O₂. After the incubation period, tissue specimens were washed three times in fresh culture medium to remove any nonadherent bacteria and were processed for scanning EM prior to examination with a JEOL JSM-5300 scanning electron microscope.

Counts of adhering bacteria for each specimen were taken from 10 random

		10	20	30	40	50	
AAFA	1	MKKIRM---	F AIATLLSSGA	--AINATAVA	KTA*STITVV	NFCDIITTPA	50
AGGA	1	MRRKNLCITL	GLVSLLSRGA	NAALERPPPIK	ATEIIRLTVT	NDCPVITATN	50
		60	70	80	90	100	
AAFA	51	TNRDVNDRS	AN*DLSTIR	QPQR*ADAGM	RIKAWGEANH	GQLLIKPGGG	100
AGGA	51	SPPNVGVSS	TP*IFNATVT	TTEQCAKSGA	EVWLWGTGAA	NKWVLE-HTT	100
		110	120	130	140	150	
AAFA	101	NKSAGETLAS	P----RFSYI	FNNPANI*MG	FVLTNPGVYQ	LGMQGSITPA	150
AGGA	101	NTKQKYTLNP	SIDGNSYFQT	EGTNAALYKN	VTTRDRV---	LKASVKVD*EK	150
		160	170	180	190	200	
AAFA	151	IP-IRPGLYE	VVLNAELVTN				200
AGGA	151	IQVLIIPGTYR	MI*HAG-INF				200

FIG. 3. BESTFIT comparison of predicted amino acid sequences derived from the genes encoding AafA and AggA. Identical amino acids (shaded) are indicated. Note that the predicted translational start of AggA differs from that previously published (38) and more closely agrees with predictions for prokaryotic signal sequences (9, 13). The lysine residue at position 31 of AafA (underlined) represents the amino terminus of the processed protein as determined by amino-terminal amino acid sequencing. Residues characteristic of Dr family adhesins (20) are also indicated (asterisks).

FIG. 4. (A) Western immunoblot of Ni-NTA column-purified polyhistidine-tagged AafA protein, reacted with anti-AAF/II antiserum. Lane a, pQE70 carrying a cloned *aafA* gene; lane b, pQE70 control. The 17- and 15-kDa proteins (sizes are indicated on the left) represent AafA with and without signal peptides, respectively, as determined by amino-terminal amino acid sequencing. (B) Immunogold transmission EM of Ni-NTA column-purified AafA-polyhistidine protein after dialysis and staining with anti-AAF/II antiserum. Bar, 50 nm.

fields at a fixed magnification of $\times 3,500$. The median number of adhering bacteria per field was calculated, and data were compared by using a two-tailed Mann-Whitney nonparametric test, in which a P of < 0.05 was the level of significance.

Nucleotide sequence accession number. The DNA sequence of the 1.7-kb cloned insert fragment has been submitted to GenBank/EMBL under accession no. AF012835.

RESULTS

Transposon mutagenesis of strain 042. It was reported previously that strain 042 expressed a very strong AA phenotype in the HEp-2 assay, described as a honeycomb pattern, and that this phenotype could be expressed in *E. coli* HB101 upon

acquisition of the 65-MDa AA plasmid (designated pAA2) (32). Strain 042 did not express the surface fimbria known as AAF/I, as determined by gene probe hybridization (33). To identify the AA adhesin of strain 042, we performed *TnphoA* transposon mutagenesis of this strain using the donor plasmid pRT733. By restriction enzyme digestion profiles, the majority of mutants were mapped to one plasmid region of approximately 2 kb. A complete description of all mutants will be reported elsewhere. One representative pAA2-borne *TnphoA* adherence-minus mutant (042::*phoA* 3.4.14) was selected for further genetic analysis. Plasmid DNA was extracted from mutant 042::*phoA* 3.4.14 and transformed into *E. coli* HB101.

TABLE 2. Mannose-resistant HA patterns of AAF/I and AAF/II isogenic strains^a

Bacterial strain	AAF ^b	HA ^c with the following erythrocytes:				
		Human	Rat	Rabbit	Bovine	Sheep
17-2	I	++	++	++	++	++
HB101(pJPN31)	I	++	+	+	++	+
HB101(pJPN32.1)	N	—	—	—	—	—
042	II	+	—	—	—	—
042::phoA 3.4.14	N	—	—	—	—	—
HB101(pAA2)	II	+	—	—	—	—
HB101(pAA2::phoA 3.4.14)	N	—	—	—	—	—
HB101	N	—	—	—	—	—

^a Overnight cultures were centrifuged, and the bacterial cells were resuspended in saline. Equal volumes of bacterial suspension and 3% erythrocyte suspension (with 1% mannose) were mixed and incubated at room temperature for 20 min before HA was examined.

^b AAF result determined by probe. N, negative with probes for AAF/I and AAF/II.

^c ++, visible with the unaided eye; +, visible only under light microscopy.

Kanamycin-resistant transformants acquiring 042::phoA 3.4.14 plasmid DNA were found to express alkaline phosphatase and to have acquired a 70-MDa plasmid species (pAA2::phoA 3.4.14); all such transformants were negative for AA, whereas HB101 harboring the native pAA2 plasmid was AA positive. Southern analysis of restricted pAA2::phoA 3.4.14 DNA with a probe consisting of the internal *Bgl*II fragment of *TnphoA* revealed that the mutant plasmid carried a single *TnphoA* insertion (not shown).

Transmission EM was performed on HB101, HB101 (pAA2), and HB101(pAA2::phoA 3.4.14). Only HB101(pAA2) exhibited surface fimbriae (Fig. 1a). These fimbriae were thicker (5-nm diameter) than those reported previously (29) for AAF/I (2- to 3-nm diameter [Fig. 1b]); the structures were more rigid than AAF/I and were found most commonly as loose bundles of filaments. We have designated these novel fimbrial structures AAF/II.

To test for immunologic relatedness between AAF/I and

AAF/II, we performed immunogold EM on strains 042 and HB101(pAA2), using anti-AAF/I antiserum as described previously (29). AAF/II fimbrial structures did not stain with anti-AAF/I antiserum (Fig. 2).

On the basis of previous observations that the plasmids of 042 and 17-2 were highly homologous (3, 43), we hypothesized that the AAF/I and AAF/II fimbrial gene clusters might exhibit close genetic homology, despite the immunologic and morphologic differences between their surface adhesins. Separate DNA fragments representing region 1 (a 6.8-kb *Cla*I fragment) and the *aggR* gene (a 1.3-kb *Pst*I-*Ssp*I fragment) of AAF/I were hybridized individually with strain 042 and with HB101(pAA2) DNA by colony blotting, under high stringency. The region 1 probe did not hybridize with 042 or HB101(pAA2), whereas the *aggR* probe yielded a strong signal suggesting a high level of homology. To support further the presence of an *aggR* homolog, the 1.3-kb *Pst*I-*Ssp*I gene fragment comprising only the cloned *aggR* gene was used as a probe in Southern analysis of *Bam*HI- and *Mlu*I-digested pAA2 DNA. Hybridization and autoradiography revealed a strong signal under high stringency for the *aggR* probe, corresponding to an *Mlu*I fragment of 20 kb and a *Bam*HI fragment of 70 kb (not shown). These data suggest that the AAF/II plasmid pAA2 carries an *aggR*-homologous sequence but not sequences identical to the other AAF/I genes.

DNA sequence determination of cloned adherence-related genes. To characterize the adherence-related locus interrupted by *TnphoA* in mutant 042::phoA 3.4.14, the *TnphoA*-flanking DNA was cloned and sequenced. A 7-kb *Bam*HI fragment containing both the *phoA* gene and flanking DNA was cloned into pBluescript II SK, and the *phoA*-flanking nucleotide sequence was determined. From this sequence, a DNA probe which hybridized with a 1.7-kb *Eco*RI fragment from pAA2 in Southern analysis was derived. The 1.7-kb fragment was cloned into pBluescript II SK (to yield pJC2); the clone did not confer HEP-2 adherence upon host strain HB101.

The DNA sequence of the 1.7-kb *Eco*RI fragment was determined in its entirety and comprised 1,720 bp. Analysis of the sequence revealed only one complete ORF, designated the *aafA* gene (nucleotides 1139 to 1618). By sequence analysis, *aafA* corresponded to the site of *TnphoA* insertion in mutant 042::phoA 3.4.14. *aafA* yielded a predicted protein product of 160 amino acid residues (17,062 Da), with a typical signal sequence at the N terminus (9, 13). The amino acid sequence included two cysteine residues at positions 38 and 70. Kyte-Doolittle hydrophobicity analysis suggested three domains: a predominantly hydrophobic region corresponding to residues 1 to 40 (including the signal sequence) and a predominantly hydrophilic domain from residues 40 to 118 followed by a predominantly hydrophobic C terminus (residues 119 to 160). This hydrophobic C terminus of the AafA product, coupled with the presence of a glycine at position 147, is consistent with predictions made for fimbrial proteins which are protected by P-fimbria-class chaperones during export (18).

BLAST analysis suggested that the closest homolog of *aafA* was the *aafA* gene (i.e., the fimbrial subunit of AAF/I in strain 17-2) (BLAST probability score = 2.9×10^{-5}). BESTFIT analysis of the *aggA* gene product compared with AggA revealed 25% identity and 47% similarity (Fig. 3). The isoelectric points of the proteins were similar (10.09 and 10.27 for AafA and AggA, respectively). Several residues typical of the Dr family of adhesins (20) were conserved in both proteins: threonine at AafA position 33, cysteine at position 38, isoleucine at position 58, cysteine at position 70, and tyrosine at position 149. The sizes and positions of the predicted disulfide loop were similar in the two proteins.

FIG. 5. Dose-dependent inhibition of HEP-2 adherence by anti-AAF/II antiserum. Twenty microliters of bacterial culture and 50 μ l of antiserum were added simultaneously to tissue culture wells containing HEP-2 cells, and the adherence assay was performed by standard methods. After 3 h of incubation, bacterial aggregates (more than four bacteria per aggregate) were counted in four fields under $\times 400$ power and expressed as mean numbers of aggregates per field. Data are the means of three such experiments plus standard deviations. The adherence of negative controls 042::phoA 3.4.14 and HB101 was negligible.

FIG. 6. Immunogold EM of strain DH5 α carrying plasmids pJPN39 Ω 4 and pJPN45, conferring the biogenesis machinery of AAF/I but lacking a functional AAF/I subunit (a), and carrying pJPN39 Ω 4 and pJPN45 as well as pJC3, which encodes the fimbrial subunit of AAF/II (b). Bars, 250 nm.

Upstream from *aafA* (nucleotide positions 1 to 710) was a partial ORF with 68% identity to the chaperone of *E. coli* nonfimbrial adhesin 1 (the *nfaE* gene [accession no. S61968]). Translation of our ORF would yield a predicted protein of 26 kDa. Comparison with the *nfaE* gene suggested that our ORF contained 85% of the gene from the 3' end and lacked the translational start and a signal sequence.

To assess the ability of the *aafA* gene to complement the *phoA* 3.4.14 Tn*phoA* insertion, the 1.7-kb *aafA* clone (pJC2) was transformed into HB101(pAA2::*phoA* 3.4.14). Upon acquisition of pJC2, HB101(pAA2::*phoA* 3.4.14) acquired the AA phenotype and the expression of surface fimbriae (data not shown).

Complementation data thus suggest that the insertion mutation in 042::*phoA* 3.4.14 abolishes fimbrial production by interruption of a fimbria-associated gene and not through polar effects. The similarity of the *aafA* gene product with the AggA protein suggests that AafA is the fimbrial subunit of the AAF/II fimbriae. To test this hypothesis, we expressed the *aafA* gene under *tac* control in the vector pQE70, which adds a polyhistidine tail to the C terminus of the AafA protein. The gene product was purified by nickel column chromatography of cell lysates. Western immunoblot analysis of these lysates with anti-AAF/II antiserum revealed the presence of two dominant proteins of 15 and 17 kDa, consistent with processed and unprocessed forms of the protein (Fig. 4A). Dialysis of the purified protein against 1 mM MgCl₂ resulted in the formation of large, poorly soluble aggregates; transmission EM of the aggregates revealed the presence of bundles of filaments, approximately 5 nm in diameter, which stained with anti-AAF/II antiserum in immunogold EM (Fig. 4B). Amino-terminal se-

quencing of the aggregated protein revealed that the predominant 15-kDa species was identical to the predicted AafA protein but lacked its predicted signal sequence. The amino acid sequence of the 17-kDa species agreed with the predicted sequence of the unprocessed AafA. The spontaneous formation of 5-nm-diameter filaments by the His-tagged AafA protein provided further support that the AafA protein is in fact the fimbrial subunit of AAF/II.

Binding properties of AAF/II. To test AAF/I and AAF/II for the ability to recognize a common receptor, HA was performed on isogenic AAF/I- and AAF/II-positive and -negative pairs; the results of these experiments are presented in Table 2. AAF/I conferred broad-host-range HA, visible with the naked eye. AAF/II conferred only weak human erythrocyte HA, discernible only by light microscopy. Strain 042 adhered strongly to polystyrene after overnight incubation, producing a thick film on the bottom of the culture plate; strain 17-2 did not exhibit detectable polystyrene adherence.

At a dilution as low as 1:32, polyclonal antiserum reacting only with AAF/II as determined by immunogold transmission EM inhibited HA of human erythrocytes by strain 042 and yielded a dose-dependent inhibition of HEP-2 adherence (Fig. 5).

The similarity of the *aggA* and *aafA* genes suggested that the respective protein products might be able to substitute for each other in the assembly of functional fimbriae and also would allow us to determine whether the differences in phenotypes attributable to the two fimbriae were conferred by their respective fimbrial subunits. To test this, a three-way complementation analysis was performed, wherein the AAF/I biogenesis cluster (region 1) was provided by clone pJPN39 Ω 4, in

FIG. 7. Scanning EM of colonic specimens incubated with EAEC strain 042 and AA-negative transposon mutant 042:*phoA* 3.4.14. Viable colonic specimens were incubated for 8 h with bacterial cultures and then washed, fixed, stained, and examined under scanning EM. (a) Strain 042 under low magnification. (b) Higher magnification of panel a, showing 042 cells adhering to the colonic mucosa. (c) Afimbrial mutant 042:*phoA* 3.4.14. Few adherent bacteria are evident. Mucosal changes, consisting of widening of crypt openings and increased mucus secretion, are evident in panels a to c compared with the uninfected colon (d). Bars, 50 (a, c, and d) and 5 (b) μm .

FIG. 7—Continued.

which the fimbrial subunit gene (*aggA*) was interrupted by the Ω interposon (introducing a transcriptional terminator). In the presence of the activator gene *aggR* (provided on chimeric plasmid pJPN45), region 1 is normally able to express AAF/I, yet fimbriae are not expressed by pJPN39 Ω 4 due to the lack of a functional *aggA* gene. pJPN39 Ω 4 and pJPN45 were introduced into *E. coli* DH5 α ; the *aafA* gene was then introduced (on plasmid pJC3), and HEp-2 adherence, HA, polystyrene adherence, and fimbrial formation were assessed. Immunogold transmission EM of the AAF/I-AAF/II heterologous construction DH5 α (pJPN39 Ω 4)(pJPN45)(pJC3) using anti-AAF/II antiserum revealed the presence of the typical filaments of AAF/II (Fig. 6), whereas DH5 α (pJPN39 Ω 4)(pJPN45) did not express fimbriae that bound anti-AAF/II antiserum. DH5 α (pJPN39 Ω 4)(pJPN45)(pJC3) adhered to HEp-2 cells in an AA pattern and demonstrated polystyrene adherence, yet the strong human HA pattern characteristic of AAF/I was not present, suggesting that the specificity of HA phenotypes is conferred by the respective fimbrial subunits.

IVOC studies. EAEC strain 042 has been shown to adhere to human intestinal explants in vitro (30). To determine the role of the AAF/II fimbriae in this phenotype, we incubated 042 and 042::*phoA* 3.4.14 with pediatric jejunal and colonic specimens for 8 h. Scanning EM of infected jejunal mucosa revealed that 042 adhered in significant numbers ($P < 0.02$ versus uninfected controls) and caused an increase in cellular extrusion (data not shown). However, strain 042::*phoA* 3.4.14 did not adhere to any specimen in numbers significantly higher than those of uninfected controls, and fewer extruding cells were seen. Scanning EM of infected colonic tissue (Fig. 7) revealed that strain 042 adhered well to all specimens but that 042::*phoA* 3.4.14 was nonadherent; the difference in counts of adherent bacteria was significant at a P of < 0.0001 . Toxic effects on the mucosa, including increased cellular extrusion, enlarged crypt openings, and rounding of the remaining cells, were seen in the 042-infected tissue but were substantially reduced in the tissue infected with the afimbrial mutant.

Prevalence of AAF/I and AAF/II. We selected 51 EAEC strains isolated in various epidemiologic studies around the world and performed colony blot analysis using the CVD432 (AA) probe (previously described [3]), a PCR-derived fragment probe representing only the *aggA* gene of AAF/I, and the 1.7-kb *EcoRI* fragment of AAF/II (described above). Thirty-six (71%) of the strains were AA probe positive. Sixteen (31%) of the strains hybridized with the AAF/I fragment, whereas six (12%) hybridized with the AAF/II fragment. No strain hybridized with both AAF/I and AAF/II probes; all strains which hybridized with either fragment were positive with the AA probe. Fourteen of the AA probe-positive strains (27% of all strains) and all of the AA probe-negative strains hybridized with neither AAF/I nor AAF/II. Five of five AAF/I probe-positive strains were positive for AAF/I expression by immunogold EM; none of six AAF/II-positive strains were stained by the AAF/I antibody. AAF/II probe-positive strains all were found to express 5-nm-diameter rigid fimbrial structures binding AAF/II antiserum.

DISCUSSION

The physical and genetic characterization of an EAEC fimbrial adhesin, designated AAF/I, which confers AA and HA of human erythrocytes, has been reported previously (28, 29, 34, 38). On the basis of data suggesting that most EAEC strains harbor a highly conserved adherence plasmid (43), we surmised that all such strains would produce AAF/I. In this series of experiments, we have found that this is not the case.

Here, we describe a second AA-conferring fimbria, designated AAF/II, which confers AA in the human-pathogenic strain 042. The data suggest that AAF/I and AAF/II are members of a new family of *E. coli* fimbrial adhesins; the distant but significant homology of AAF/I to the Dr family suggests that the AAF family may be a subfamily of Dr adhesins. Despite the relatedness of the AAF subunits, it is significant that the accessory genes do not display sufficient identity to permit hybridization on Southern analysis, since within fimbrial families accessory genes are generally more highly conserved than fimbrial subunits (19, 35). In addition, not only is the fimbrial subunit of AAF/II distinct from that of AAF/I, but the organization of the fimbrial biogenesis genes is apparently different as well: AAF/I features the organization typical of Dr family adhesins (chaperone-usher-cryptic gene-subunit), whereas the AAF/II cluster features a chaperone homolog immediately upstream of the fimbrial subunit gene. Indeed, studies in our laboratory suggest the presence of a unique organization for AAF/II biogenesis genes.

Colony hybridization data suggest that among the AA probe-positive strains, there are AAF/I producers and AAF/II producers and some strains which express neither. This implies the presence of an AAF/III (and possibly still more adhesins) which may be carried on the AA plasmid. Moreover, EAEC strains are apparently similar to enterotoxigenic *E. coli* strains in that adherence can be conferred by one of several different fimbrial antigens. Whereas some EAEC isolates are clearly human pathogens (33, 39), it is likely that not all members of this category are virulent. Our AAF/I and AAF/II gene probes may be useful for the subdivision of EAEC strains in epidemiologic analyses.

An unusual, conserved feature of the AAF/I and AAF/II fimbrial subunits is their high isoelectric points (10.3 and 10.1 for AggA and AafA, respectively). This stands in contrast to the isoelectric points of Dr family members (approximately 6.0) and those of most other fimbrial subunits (26). This high isoelectric point may result in a net positive charge to the surface of EAEC strains at physiologic pH, a phenomenon which has been linked to adherence to glass and to biofilm formation (14). An alternative, or perhaps additional, explanation for the aggregative nature of AAF-mediated adherence may be the property of surface hydrophobicity. Our data suggest that both AAF/I and AAF/II confer increased surface hydrophobicity on their respective host strains (26). Studies are under way to test these hypotheses.

EAEC strain 042 adheres to human intestinal explants and induces toxic effects (30); a plasmid-cured 042 derivative was unable to adhere and unable to elicit toxicity. Here, we show that an isogenic afimbrial mutant is unable to adhere to the explanted tissue and that toxic effects, while still present, are markedly less. These data suggest that the cytotoxin of strain 042 is encoded by the AA plasmid and that the cytotoxicity is augmented by the proximity of closely adhering, toxin-producing organisms. Thus, the IVOC assay suggests a paradigm for EAEC pathogenesis in which AAF-mediated colonization is followed by the elaboration of cytotoxins. Future studies will test this hypothetical model.

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