Aggregative Adherence Fimbriae I of Enterohaemorrhagic Escherichia coli Mediate Adherence to HEP-2 Cells and Haemagglutination of Human Erythrocytes

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Received 30 January 1992/Accepted 30 March 1992

Strains of enterohaemorrhagic Escherichia coli (EAggEC) have been implicated in several studies as important agents of persistent diarrhoea among infants in the developing world. We have previously shown that the aggregative adherence (AA) property of EAggEC is associated with the presence of a 60-MDa plasmid which confers AA when introduced into E. coli HB101. Here, we report the cloning of the AA determinant from EAggEC strain 17-2 into the 21.5-kb cosmid vector pCVD301. TnphoA mutagenesis of the AA cosmid clone pJPN31 implicated an AA region of approximately 12 kb. Transmission electron microscopy of HB101 (pJPN31) revealed the presence of bundle-forming fimbriae, which were absent in AA− TnphoA insertion mutants. The presence of these fimbriae, AA, and haemagglutination (HA) of human erythrocytes were all concurrently lost by single-insertion mutations. A 14-kDa protein was seen on polyacrylamide gel electrophoresis and Western blotting (immunoblotting) of surface shear preparations from fimbriated clones. Twelve of nineteen volunteers fed EAggEC 17-2 developed rises in antibodies to the 14-kDa protein as determined by Western blot. We have termed the cloned bundle-forming fimbriae aggregative adherence fimbria I (AAF/I); positivity with a previously described EAggEC probe and human erythrocyte HA appear to correlate with the presence of AAF/I.

MATERIALS AND METHODS

Strains. Wild-type E. coli strains used in this study are from the collection of the Center for Vaccine Development. These isolates originate from various sites around the world, including Santiago, Chile; Lima, Peru; Armed Forces Research Institute of Medical Sciences, Thailand (courtesy of Peter Echeverria); Israel (courtesy of Dani Cohen); and Guadalajara, Mexico (courtesy of John Mathewson). The prototype EAggEC strain used for this study is E. coli 17-2 (serotype O3:H2), isolated from the diarrheal stool of an infant in Santiago, Chile (33). This patient experienced watery diarrhoea of unknown duration without gross blood in the stool. 17-2 harbors a 60-MDa plasmid (p17-2) which, when transformed into E. coli HB101, confers AA (31) and the production EAST (26).

Genetic manipulations were performed with E. coli HB101 (merB mrr hsdS29 recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20[S^m] supE44) (5). HB101 is rough and produces few type 1 pili. TnphoA4 carried on plasmid pRT733 was maintained in the pir-lysogenized strain E. coli SM10pir as previously described (27, 30).

All strains were propagated on Luria agar (L agar) or in L broth at 37°C and were stored at −70°C in L broth supplemented with 15% glycerol. Selection for antibiotic resistance was performed on L-agar plates with the following antibiotic concentrations: tetracycline, 30 μg/ml; ampicillin, 100 μg/ml; kanamycin, 50 μg/ml.

Molecular cloning. Plasmid DNA for large- and small-scale preparations was extracted by the alkaline lysis method (1).
Large-scale plasmid preparations were further purified by cesium chloride-ethidium bromide isopycnic ultracentrifugation in a Beckman L8-80 ultracentrifuge. DNA was analyzed by agarose gel electrophoresis. Purification of DNA fragments and extraction from gel slices were performed with Geneclean (Bio 101, La Jolla, Calif.). Plasmid DNA was introduced into E. coli HB101 by transformation of competent cells obtained from GIBCO/BRL (Gaithersburg, Md.) according to the method of Hanahan (13).

Restriction enzymes and T4 DNA ligase (GIBCO/BRL) were used according to the manufacturer’s instructions. In order to construct a gene library of AA plasmid p17-2, plasmid DNA was partially digested with Sau3A to yield 15- to 30-kb fragments, which were separated and extracted from a 0.7% agarose gel. The fragments were ligated with BamHI-digested pCVD301, a 21.5-kb low-copy-number cosmid derivative of RK290 which confers resistance to tetracycline (9). Ligated DNA was packaged into phage by using the Gigapack II system (GIBCO/BRL) and was transfected into HB101.

Transposon mutagenesis. Transposon mutagenesis was performed on a single representative AA cosmid clone, pJPN31, by using TnphoA. TnphoA is a Tn5-derived transposon which encodes kanamycin resistance and the carboxy terminus of E. coli alkaline phosphatase (PhoA) (18). Transposition mutants will express PhoA if fusion occurs in frame with a gene for an exported bacterial polypeptide. For these experiments, TnphoA was delivered on the suicide plasmid pRT733, whose R6K replicon is dependent upon the \( \pi \) protein for stable replication. The plasmid replicates in E. coli SM10\( \alpha \)pir, which carries the genes encoding \( \pi \) on lysogenized lambda sequences and also permits mobilization of the plasmid via RP4 tra genes (27, 30). pRT733 was mobilized into HB101(pJPN31) by filter mating. Twenty-microliter volumes of 4-h donor and recipient cultures were mixed on cellulose nitrate membranes (0.45-\( \mu \)m pore size; Nuclepore Corp., Cambridge, Mass.) overlying L-agar plates and incubated overnight. The bacterial growth on the membrane was then suspended in 1 ml of LB broth containing kanamycin (30 \( \mu \)g/ml) and tetracycline (15 \( \mu \)g/ml) and incubated at 15 rpm at 37°C. Plasmid DNA extracted from this culture was transformed into HB101, selecting for tetracycline and kanamycin resistance. The PhoA-expressing transposon insertion mutants was assayed by inoculating L-agar plates containing 30 \( \mu \)g of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Sigma Chemical, St. Louis, Mo.) per ml.

HA. HA was performed with human type A erythrocytes as described by Yamamoto et al. (37). Briefly, E. coli was grown overnight in static L-broth cultures, pelleted in an Eppendorf 4515C microcentrifuge at 10,000 \( \times \) g for 1 min, washed twice in sterile phosphate-buffered saline (PBS), and resuspended in PBS to a concentration of 10^9/ml (estimated with a 10× concentrate by McFarland nephelometry [16] and confirmed by plate counts). Serial twofold dilutions of the bacterial suspension in PBS were prepared; 100 \( \mu \)l of each dilution was mixed with an equal volume of a 3% (vol/vol) washed human type A erythrocyte suspension containing 1% D-mannose (Sigma Chemical) in PBS. The suspensions were briefly mixed in a 24-well tissue culture dish and allowed to stand at room temperature for 20 min. After this time, HA was evaluated under \( \times 400 \) magnification with an inverted microscope. Any HA discerned to be greater than that of a negative bacterium-free control was considered to be positive; the highest dilution of the bacterial suspension producing HA was recorded for each strain tested.

Inhibition of HA was performed by incubating serial twofold dilutions of antiserum in PBS with an equal volume of a washed bacterial suspension (10^9/ml) at room temperature for 20 min. A 100-\( \mu \)l portion of this preparation was mixed with 100 \( \mu \)l of the 3% erythrocyte–1% mannose suspension, and HA was assayed as described above.

HEp-2 adherence assay. The HEp-2 adherence assay was performed by the CVD method (33), based on the initial protocol described by Cravitti et al. (7). HEp-2 cells were grown overnight to 50% confluent monolayers on glass coverslips in 24-well tissue culture dishes. Spent medium was discarded, and 20 \( \mu \)l of overnight L-broth bacterial culture plus 1 ml of fresh Eagle’s minimal essential medium (GIBCO/BRL) with 0.5% D-mannose was added to each well. The assay was incubated for 3 h at 37°C in 5% CO wellbeing. After incubation, cells were washed twice with PBS, fixed with 70% methanol for 5 min, and then stained with 10% Giemsa for 15 min. Coverslips were examined under oil immersion light microscope for the characteristic stacked brick aggregation on HEp-2 cells and on the glass coverslip. All positive specimens displayed at least one aggregate of more than five bacteria per high-power field. This method, featuring a 3-h incubation without a change of tissue culture medium, has been shown to be optimal for the detection of the aggregative phenotype (33).

DNA colony hybridization. We performed colony hybridization on a select group of E. coli strains, using the EAggEC probe described by Baudry et al. (2). Blots were prepared by the colony lift technique previously published (11). A 100-ng portion of the 1-kb probe fragment was labelled with random primer (Prime-it Kit; Stratagene Cloning Systems, La Jolla, Calif.) and 50 \( \mu \)Ci of [\(^{32}\)P]dATP (Amersham Life Science Products, Arlington Heights, Ill.).

Volunteer studies. Serum samples from 19 adult volunteers who ingested 10^{10} EAggEC 17-2 organisms with sodium bicarbonate buffer were taken before and 21 days following challenge. The methods for recruitment of volunteers, clinical surveillance, and bacteriology followed previously published procedures (29). The clinical protocol was approved by the Institutional Review Board of the University of Maryland at Baltimore. Results of the clinical studies will be reported in detail in a subsequent publication (28).

Shear preparation of Western blots. Cell suspensions were disrupted by mechanical shearing of intact bacterial cells. One milliliter of an overnight L-broth culture was adjusted to 3 \( \times \) 10^8 bacteria per ml by McFarland nephelometry (16). One milliliter of this suspension in a 1.5-ml Eppendorf tube was subjected to vortexing in a VWR Vortex Genie (highest speed) for 5 min. Bacterial cells were pelleted at 10,000 \( \times \) g for 1 min in an Eppendorf 4515C microcentrifuge. Supernatants were separated by 18% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (15), and proteins were visualized by silver staining (Bio-Rad Laboratories, Richmond, Calif.). Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) by the method of Towbin et al. (31). Blots were blocked in PBS-0.5% Tween 20 (PBST) for 20 min and then incubated overnight at 4°C with primary antiserum diluted in PBST. Blots were washed three times for 10 min each in PBST and incubated for 2 h in a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G or anti-human immunoglobulin G (Cappel Research Products, Durham, N.C.) in PBST. Binding of antibody was detected with BCIP (165 \( \mu \)g/ml) and nitroblue tetrazolium (330 \( \mu \)g/ml) (NBT; Sigma Chemical) for 10 min at room temperature (14).
A surface shear preparation of HB101(pJPN31) was separated by 18% SDS-PAGE using a trough preparative comb, and the gel was transferred to nitrocellulose paper. Thin strips representing identical antigen preparations were cut from this blot for the analysis of volunteer antisera.

Antiserum preparation. New Zealand White male rabbits (1.5 kg) were inoculated intravenously with live bacteria in doses escalating from $10^6$ to $10^{10}$ organisms at 3-day intervals for a total of 12 doses and then boosted intravenously with $10^{10}$ organisms 3 weeks later. Each inoculum was prepared fresh by harvesting overnight cultures of HB101(pJPN31) at 10,000 $\times g$ in an Eppendorf 4515C microcentrifuge for 1 min, washing them twice with PBS, and resuspending the pellet in 1.0 ml of sterile PBS at the appropriate concentration (determined by McFarland nephelometry). Two weeks after the completion of the immunization series, rabbits were bled to obtain serum.

Antiserum raised against HB101(pJPN31) was exhaustively absorbed with the TnphoA insertion mutant HB101 (pJPN32.26) (negative for AA, HA, autoagglutination, and PhoA expression). An HB101(pJPN32.26) overnight culture was harvested and resuspended in PBS to a concentration of approximately $10^{12}$ cells per ml. HB101(pJPN31) antiserum (1 ml) was diluted 1:4 in PBS and then incubated with the bacterial suspension at 4°C with gentle shaking; the absorption was repeated until only faintly reactive bands were seen on Western blots of HB101(pJPN32.26) surface shear preparations, and no further diminution was produced by subsequent absorptions.

Volunteer sera were absorbed with E. coli HB101 lipooligosaccharide (LOS) antigen extracted by the hot water-phenol method (35). Nitrocellulose paper strips were saturated in 15 $\mu$g of LOS per ml of water and then preincubated for 2 h with volunteer sera diluted 1:100 in PBS. Two milliliters of the absorbed 1:100 dilution was then used as the primary antiserum in Western blot reactions of the HB101 (pJPN31) shear preparation strips described above.

To test for serologic cross-reactivity of HB101(pJPN31) antigens with other fimbrial colonization factors of E. coli, we performed Western blot analysis of HB101(pJPN31) shear preparations by using specific polyclonal antisera against enterotoxigenic E. coli fimbriae CFA/I, CS1, CS2, CS3, CS4, CSS, CS6, and PCFO159:H4 and also against rigid fimbriae extracted from intact 17-2 (34) and enterohemorrhagic E. coli 933. The fimbrial antisera were prepared by the subcutaneous inoculation of rabbits with purified fimbriae according to published methods (14, 17). Each antiserum was employed at a dilution appropriate to detect the homologous antigen.

**Electron microscopy.** Transmission electron microscopy of negatively stained specimens was performed by standard methods (17) on a JEOL JEM 1200 EX II transmission electron microscope.

**RESULTS**

Cloning of the AA factor. A gene library of p17-2 was constructed in the cosmid vector pCVD301. HB101 harboring cosmid clones displayed one of three possible phenotypes in the HEP-2 assay: (i) nonadherence, (ii) predominantly “specific” AA (SAA) to HEP-2 cells, and (iii) AA to the glass coverslip and to HEP-2 cells equally well (nonspecific AA [NAA]). SAA clones, but not NAA clones, produced very strong autoagglutination in static L-broth cultures, easily visible with the naked eye.

Since wild-type 17-2 displays SAA in the HEP-2 assay, we chose the SAA clones for detailed analysis. The eight SAA clones were restriction mapped and were found to have a common region of approximately 12 kb, suggested by the presence of five common BglII fragments. One typical SAA clone, designated pJPN31, was mapped for the recognition sites of the enzymes SalI, Smal, MluI, and BamHI (Fig. 1).

**TnphoA mutagenesis of AA clone pJPN31.** pJPN31 was subjected to mutagenesis with TnphoA (see Materials and Methods). When tested in the HEP-2 assay, 86 insertion mutants (designated the pJPN32 series) were found to have lost the SAA phenotype. Analysis of the sites of transposon insertion implicated a 12-kb AA region, indicated in Fig. 1. Insertion into one site within the AA region was accompanied by a change in the adherence phenotype to NAA. Three different nonadherent TnphoA insertion mutations expressed alkaline phosphatase activity. All insertion mutants which lost SAA, changing to either NAA or nonadherence, lost the property of autoagglutination in broth cultures. One particular nonadherent TnphoA mutant, pJPN32.26, was chosen as the representative nonadherent strain. A second TnphoA mutant, pJPN32.2, was chosen as the representative NAA strain. Both pJPN32.26 and pJPN32.2 were negative for alkaline phosphatase activity.

**Demonstration of AA fimbriae by electron microscopy.** Since AA in the HEP-2 assay is consistently demonstrated by using static L-broth cultures, bacterial samples for electron microscopy examination were grown in this manner. When HB101(pJPN31) was examined by transmission electron microscopy, long, bundle-forming fimbriae were visualized (Fig. 2). These fimbriae typically connected bacteria together in aggregates. Individual filaments were occasionally observed and appeared flexible, with a diameter of

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**FIG. 1.** Map of AA clone pJPN31. pJPN31 consists of a 29-kb insert from p17-2 cloned into the 21.5-kb cosmid vector pCVD301 (heavy line). pJPN31 confers AA, HA, and fimbrial production in HB101. Insertion of TnphoA into the region marked by the solid bar abolishes AA. The hatched area indicates the position of TnphoA insertions which change the phenotype from SAA to NAA (see text). The region required for AA expression has been designated aaf for aggregative adherence fimbriae.
FIG. 2. Transmission electron photomicrograph of negatively stained HB101(pJPN31). Flexible fimbriae (2-nm diameter) are seen extending between bacteria. A typical bundle is seen in the lower portion of the photomicrograph. These fimbriae are not seen in HB101(pJPN32.26), a TnphoA insertion mutant which lacks AA and HA, nor are they seen in HB101(pCVD301).
were visualized with band was not the HB101(pJPN32.26) HB101(pJPN32.2), HB101(pCVD301) 17-2

<table>
<thead>
<tr>
<th>Organism</th>
<th>HEP-2 adherence phenotype</th>
<th>Highest dilution producing HA</th>
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<tbody>
<tr>
<td>HB101(pJPN31)</td>
<td>SAA</td>
<td>1:64</td>
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<tr>
<td>HB101(pJPN32.26)</td>
<td>Nonadherence</td>
<td>0</td>
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<tr>
<td>HB101(pJPN32.2)</td>
<td>NAA</td>
<td>1:2</td>
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<tr>
<td>HB101(pCVD301)</td>
<td>Nonadherence</td>
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<td>17-2</td>
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approximately 2 nm. Such fimbriae were not seen on HB101 or on HB101 harboring AA' TnphoA insertion mutation pJPN32.26.

HA of AA clones. We performed quantitative HA on 17-2, HB101, HB101(pJPN31), and the TnphoA insertion mutants HB101(pJPN32.26) (nonadherent) and HB101(pJPN32.2) (NAA) (Table 1). HB101(pJPN32.26), which displayed loss of fimbriae upon electron microscopy, loss of autoagglutination in broth culture, and loss of AA in the HEP-2 assay, also lacked HA activity. NAA mutant pJPN32.2 conferred greatly diminished HA. HA was not expressed in cultures grown on CFA (colonization factor antigen) agar or L agar. 17-2 grown in broth culture after multiple passages on L-agar plates again expressed HA.

Western blot analysis of AA fimbria. Polyclonal antifimbrial serum raised against HB101(pJPN31) and absorbed with HB101(pJPN32.26) completely inhibited HA by HB101 (pJPN31) at a dilution of 1:32; preimmunization antiserum did not inhibit HA. Surface shear preparations of HB101 (pJPN31) and HB101(pJPN32.26) were separated by SDS-PAGE (Fig. 3). The predominant protein band of HB101 (pJPN31) had an apparent molecular mass of 14 kDa, yet this band was not visualized in HB101(pJPN32.26) preparations. Surface shear preparations were separated by SDS-PAGE, transferred to nitrocellulose paper, and reacted with the antifimbrial serum. The 14-kDa protein was again the predominant band in Western blots of HB101(pJPN31) preparations; the intensity of this band was greatly diminished in shear preparations of HB101(pJPN32.26), and the band was absent in HB101(pCVD301) shear preparations (Fig. 4). The 14-kDa band was present in HB101(pJPN32.2) preparations (data not shown).

FIG. 4. Western blot analysis of EAggEC strains. Surface shear preparations from overnight L-broth cultures were separated by 18% SDS-PAGE and transferred to nitrocellulose. The blot was reacted with a 1:1,000 dilution of antiserum raised against HB101 (pJPN31) and absorbed with HB101(pJPN32.26). (A) HB101 (pJPN31); (B) HB101(pJPN32.26); (C) 17-2; (D) 042; (E) 069; (F) 133; (G) JM221; (H) HB101(pCVD301). The 14-kDa band is indicated.

FIG. 5. Western blot analysis using volunteer pre- and postchallenge sera. A surface shear preparation of HB101(pJPN31) was separated by 18% SDS-PAGE using a preparative trough comb; the gel was transferred to nitrocellulose by electroblotting, and the paper was cut into strips. Each strip was reacted with a different primary antiserum at a dilution of 1:100 in PBST. (A) Pre- and postimmunization rabbit serum; (B to E) pre- and 21-day-postchallenge pairs of volunteer antisera. The 14-kDa band is indicated.
wild-type EAggEC strains exhibited the 14-kDa band and, in addition, a smaller band of approximately 12 kDa which reacted with the rabbit antifimbrial serum but was very faint in HB101(pJPN31). One AA + HA - EAggEC strain, 069, exhibited a faintly reactive band which was slightly larger than that of the 14-kDa protein.

We reacted HB101(pJPN31) shear preparations with monospecific polyclonal antisera directed against several previously described enterotoxigenic E. coli fimbriae. We also employed antisera against a previously described putative fimbrial antigen of EAggEC strains (24) (a generous gift of D. C. Old) and against the 16-kDa subunit of the rigid EAggEC fimbriae described by Vial et al. (34). These antisera failed to react with the 14-kDa protein (data not shown).

Detection of antibodies in volunteers fed 17-2. Only 1 of 19 volunteers who were fed 10⁶ EAggEC 17-2 organisms experienced significant diarrhea, as evidenced by a liquid stool volume of 572 ml over 3 days. Nitrocellulose strips on which were immobilized HB101(pJPN31) shear preparations were separately incubated with prechallenge and 21-day-postchallenge serum samples from all 19 volunteers at a dilution of 1:100. Each sample was tested before and after absorption with immobilized HB101 LOS preparations. Figure 5 shows typical results of these Western blots. Before challenge, 13 of the 19 volunteers had evidence of antibodies that recognized the 14-kDa protein; 16 had antibodies after challenge. Nine serum samples which were positive before challenge showed marked increases in the intensity of the reaction to the 14-kDa band. The one volunteer who developed diarrhea had no anti-14-kDa antibody before challenge but showed a marked seroconversion detectable in postchallenge serum. Absorption of volunteer antiserum with HB101 LOS did not diminish the intensity of the reaction to the 14-kDa band. As a control, the sera of two volunteers who reacted to the 14-kDa protein were incubated with blots containing HB101(pJPN32.26) and HB101(pCVD301) shear preparations. There was no detectable 14-kDa protein in the Western blots of these preparations.

Correlation of HA with EAggEC probe positivity. The EAggEC probe was initially shown to have 89% sensitivity for EAggEC isolates from Santiago, Chile (2). In order to correlate probe positivity with HA of type A human erythrocytes, we analyzed 52 EAggEC strains of various serotypes from several sites around the world. We chose 36 which were positive with the EAggEC probe and 16 which were negative; 35 of these strains showed HA with human erythrocytes. HA of human erythrocytes correlated strongly with probe positivity (P < 0.0001 by Fisher’s exact test). Of the 36 probe-positive strains, 34 were positive for HA; of the 16 probe-negative strains, 15 were HA - . The probe was therefore 97% sensitive and 88% specific for detecting hemagglutinating strains.

DISCUSSION

The pathogenic mechanism by which EAggEC strains cause acute and persistent diarrhea is as yet unclear, although several features are beginning to emerge. Vial et al. demonstrated a characteristic intestinal histopathology in the rabbit ligated intestinal loop which included prominent adherence of the bacteria to the mucosa with hemorrhage and disruption of the villous architecture (34). The dramatic adherence of these organisms to each other and to the mucosal surface may contribute to their persistence in the human intestine; the hemorrhagic nature of the lesion is consistent with the fact that many children infected with EAggEC experience bloody diarrhea (4).

We have previously reported that AA in EAggEC is plasmid associated (23). Here, we provide data to suggest that the plasmid of 17-2 encodes bundle-forming fimbriae, which confer HA of human erythrocytes. Two observations provide evidence that these fimbriae are related to AA in 17-2. First, cloning of the AA phenotype and expression in HB101 is accompanied by electron microscopic visualization of these fimbriae. Second, TnphoA insertion in one region of the cloned DNA simultaneously abolishes AA, HA, autoagglutination in broth, and the expression of the fimbriae under the electron microscope.

Baudry et al. developed a DNA probe to detect EAggEC consisting of a 1-kb fragment from the 17-2 plasmid; this probe was initially reported to have a sensitivity of 89% and a specificity of 99% when compared with AA in the HEp-2 assay (2). The correlation of EAggEC probe positivity with human erythrocyte HA suggests that the probe is highly sensitive and specific for the presence of a single conserved adhesin. The fimbriae we have cloned likely represent this conserved adherence factor, which we term aggregative adherence fimbriae 1 (AAF/I). Two EAggEC strains were probe positive but HA negative. This may be due to the lack of expression of the aaf genes, as has been demonstrated for the diffuse adherence factor FI845 (20). Of note, strain JM221, which was shown by Mathewson et al. to be pathogenic in volunteers (19), has been recognized to be an EAggEC strain (33). In this study, we have found that JM221 is probe and HA positive and also expresses the 14-kDa protein.

Our data suggest that a large proportion of EAggEC strains express AAF/I, as defined by HA and probe positivity. However, we have noted that the probe sequences derived by Baudry et al. (2) do not lie within the aaf region described here (21). This suggests that plasmid p17-2 represents a family of conserved EAggEC plasmids, as suggested by Vial et al. (34). Some EAggEC strains apparently exhibit AA by a different adhesin.

Insertion mutagenesis indicates that a region of approximately 12 kb (designated the aaf region) is necessary for the expression of AAF/I. This region encodes multiple genes, suggested by the fact that subclones carrying only segments of the aaf genes permit trans-complementation of some TnphoA insertion mutants (21). This is consistent with observations concerning other E. coli fimbriae, which require several genes for full expression (10). The fact that the AA phenotype is altered but not lost upon insertion into one particular locus is also consistent with previous observations concerning E. coli fimbriae. Some accessory fimbrial genes are involved in the regulation, transport, or stabilization of fimbrial production (10); the absence of such genes may modify fimbrial expression without completely abolishing synthesis.

In the first description of the AA phenotype, published by our group, we highlighted the adherence of bacteria on the glass coverslip away from the surface of the HEp-2 cells. Subsequently, we have observed that many EAggEC strains adhered more prominently to the cell itself but were still in the stacked brick configuration typical of EAggEC. This cell-specific pattern is typified by 17-2. Since the probe was derived from 17-2, we have seen that both SAA and NAA may be probe positive (21). The change in AA phenotype from SAA to NAA upon TnphoA insertion into pJPN31 suggests that a difference in the expression of certain aaf genes may explain the variation in the AA phenotype.
PAGE and Western blot analyses reveal the presence of a 14-kDa protein which is expressed by HB101(pJPN31) but to a dramatically lesser degree by an A tumor-transforming mutant, HB101(pJPN32.26). We conclude that this protein is probably the AAF/I fimbrial subunit because (i) the 14-kDa band is the predominant protein on shear preparations and is the appropriate size for a fimbrial subunit, (ii) the band is markedly decreased in TnphoA insertion mutants which lose AA and HA, (iii) antibodies against this band are not lost after absorption with HB101(pJPN32.26), (iv) the 14-kDa band is present in Western blots of the EAggEC parent 17-2, (v) polyclonal antisera against the 14-kDa band inhibits HA, and (vi) most volunteers fed the 17-2 parent mount responses to the 14-kDa band. The 17-2 wild-type parent also exhibits a second protein, smaller than 14 kDa, which reacts with antifimbrial serum. This may represent the product of the processing of the fimbrial subunit, as has been reported for the toxin-coregulated pilus of Vibrio cholerae (25). HB101(pJPN31) exhibits this smaller protein, but to a lesser degree than the parent.

Experiments with volunteer sera yielded potentially important results. Despite the fact that most volunteers did not exhibit significant diarrhea upon inoculation with 17-2, 12 of 19 showed rises in the level of antibody against the 14-kDa band after inoculation. Although Western blots are only semiquantitative, the uniform antigen preparation and the standard dilution of sera allowed us to draw inferences from marked differences in reaction intensity. The observation that 13 of 19 volunteers had preexisting antibodies suggests that the U.S. population may acquire immunity to AAF/I at an early age or may have antibodies against a cross-reacting antigen, a fact which may have contributed to the low attack rate. Evidence for a possible cross-reacting antigen is provided by the fact that EAggEC 069 and several other AAF/I-negative strains express a band on Western blots which reacts with the AAF/I antiserum despite being negative to the EAggEC probe and for HA. The morphology of AAF/I is somewhat similar to that of other bundle-forming fimbiae, including the bundle-forming pilus of enteropathogenic E. coli and the toxin-coregulated fimbiae of V. cholerae. A group of fimbiae that includes these two structures, the so-called type 4 fimbiae, share antigenic cross-reactivity (25). Whether or not AAF/I belongs to this group is yet to be determined.

Most diarrheagenic E. coli fimbiae are optimally expressed on agar plates, most notably on CFA agar. Given that screening for fimbiae is generally done by using CFA agar-grow cells, new structures may be discovered when investigators examine organisms grown under other conditions. This approach led to the elucidation of the structure of the bundle-forming pilus (12). Here, we have reproduced the observations of Yamamoto et al. (37), who demonstrated that HA in EAggEC was maximal in L-broth cultures; we believe that this is due to enhanced expression of AAF/I in this medium. The expression of type 1 fimbiae is also maximal in L broth, yet the morphology of AAF/I is strikingly different.

Despite the fact that previous studies linking EAggEC to diarrhea have defined the organisms by the HEp-2 assay (3, 4, 8, 22), we believe that AAF/I producers are likely to be important pathogens, since they have represented ca. 90% of the EAggEC in studies from Chile and India (21). Insufficient data exist to determine whether or not probe-negative EA- ggEC strains are pathogenic. We are conducting further studies on the genetics, structure, and immunogenicity of AAF/I in the hope of using this structure as an immunogen in future vaccine constructions.

ACKNOWLEDGMENTS

We thank Yu Lim and Faridah Firoozmand for technical assistance and Becky Wade for electron microscopy. We also thank James B. Kaper and Robert Hall for helpful discussions.

This work was supported by grant N01-AI-15096 from the National Institutes of Health. J.P.N. is a recipient of the Norwich-Eaton/Infectious Diseases Society of America Young Investigator Award.

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