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Aggregative Adherence Fimbria I Expression in Enteroaggregative *Escherichia coli* Requires Two Unlinked Plasmid Regions

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Adherence to HEp-2 cells by many enteroaggregative *Escherichia coli* (EAggEC) strains is associated with the expression of flexible, bundle-forming fimbriae 2 to 3 nm in diameter, designated aggregative adherence fimbriae I (AAF/I). We have previously reported the molecular cloning and Tn*phoA* mutagenesis of AAF/I genes from the large plasmid of prototype EAggEC strain 17-2 (J. P. Nataro, Y. Deng, D. R. Maneval, A. L. German, W. C. Martin, and M. M. Levine, Infect. Immun. 60:2297–2304, 1992). Here, we report that further mapping and subcloning of AAF/I regions suggest that expression of the fimbriae requires two separate plasmid regions (designated regions 1 and 2). Approximately 9 kb of DNA unnecessary for fimbrial expression separates the two regions; this intervening segment encodes the EAggEC heat-stable enterotoxin (EAST1). Neither region was capable of conferring aggregative HEp-2 adherence (AA) when cloned individually; when the two regions were cloned as a single fragment or when each was cloned into a different vector and introduced into the same *E. coli* HB101 cell, AA was restored. AA-positive constructs also expressed human erythrocyte hemagglutination, autoagglutination in broth cultures, and the production of AAF/I as detected by immunogold electron microscopy.

Enteroaggregative *Escherichia coli* is a category of diarrheagenic *E. coli* implicated as an agent of persistent pediatric diarrhea in developing nations (2, 3, 8). These organisms are defined by their distinctive aggregative adherence (AA) to HEp-2 cells in cell culture (20). The AA phenotype of prototype enteroaggregative *E. coli* strain 17-2 is associated with the presence of a 60-MDa plasmid, which encodes bundle-forming fimbriae that are 2 to 3 nm in diameter (19); we have designated these structures aggregative adherence fimbriae I (AAF/I). In addition to AA, AAF/I are also associated with hemagglutination (HA) of human erythrocytes and autoagglutination in broth cultures (19).

In addition to AAF/I genes, the 60-MDa plasmid p17-2 also encodes a heat-stable enterotoxin designated EAST1 (21). Savarino and Guerry (22) have found that the gene encoding EAST1 (*astA*) is adjacent to an AA-associated locus (*aggA*). Here, we demonstrate that *astA* is in fact next to a cluster of AAF/I genes but that expression of the fimbriae also requires a plasmid locus physically removed from this cluster.

The prototype enteroaggregative *E. coli* strain used for this study, *E. coli* 17-2 (serotype O3:H2), was isolated from the diarrheal stool of an infant in Santiago, Chile. Genetic manipulations were performed in *E. coli* HB101, which expresses few type 1 fimbriae (4). All strains were propagated on Luria agar (L agar) or 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 containing 30 μg of tetracycline, 100 μg of ampicillin, or 50 μg of kanamycin per ml. All molecular cloning protocols followed standard methods (1). Tn*phoA* mutagenesis of AAF/I genes was described previously (19). Vectors and plasmids used in this study are listed in Table 1.

Hemagglutination was performed with human type A erythrocytes as described by Yamamoto et al. (25). The HEp-2 adherence assay was performed by the CVD method (23), on the basis of the original protocol of Cravioto et al. (7a). Adherence was assayed by light microscopy after a 3-h incubation at 37°C.

Transmission electron microscopy of negatively stained specimens was performed by standard methods (17) on a JOEL JEM 1200 EX II transmission electron microscope. AAF/I antiserum was prepared by intravenous injection of a viable AAF/I-positive clone in HB101, followed by exhaustive absorption with an AA(−) Tn*phoA* insertion mutant, as we have previously described (19). Immunogold electron microscopy was performed by standard methods (17).

We have previously identified a 14-kDa protein which is the predominant protein species in shear preparations of AA-producing clones (19). The amino terminus of the protein was sequenced in order to derive an oligonucleotide probe to the corresponding structural gene. HB101(pJPN31) broth culture was subjected to shearing by vigorous vortexing and pelleted by centrifugation, and the supernatant was electrophoresed in multiple lanes in 8 to 25% total acrylamide gradient sodium dodecyl sulfate gels with the Phast system (Pharmacia LKB, Piscataway, N.J.). The 14-kDa protein was electroblotted onto polyvinylidene difluoride membranes as described previously (12) with modifications to be detailed elsewhere (11). The blotted protein bands were sequenced by automated Edman degradation on a model 477A protein sequencer (Applied Biosystems, Foster City, Calif.) equipped with a model 120A analyzer. Oligonucleotides were synthesized according to manufacturer's proto-

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TABLE 1. Vectors and constructions used in this study

Vector or construction	Description[a]	Reference or source
Vectors		
pCVD301	21.5-kb cosmid with RK290 replicon (Tc)	9
pRK415	10-kb RK290-derived vector derivative (Tc)	16
pACYC177	3.9-kb low-copy-number vector (Km Ap)	New England Biolabs
pBluescript II SK	2.9-kb high-copy-number phagemid (Ap)	Stratagene
Constructions		
pJPN31	29-kb insert derived from p17-2 cloned into *Bam*HI site of pCVD301, AA(+) (Tc)	This study
pJPN33	9.4-kb *Sal*I fragment of pJPN31 cloned into pACYC177 AA(−) (Ap)	This study
pJPN35	pJPN31 with 1-kb *Xba*I fragment deleted, AA(+) (Tc)	This study
pJPN36	17-kb *Kpn*I fragment of pJPN31 cloned into pRK415, AA(−) (Tc)	This study
pJPN37	Six contiguous *Cla*I fragments of pJPN31 cloned into *Cla*I site of pACYC177, AA(+) (Ap)	This study
pJPN41	6.8-kb *Cla*I fragment of pJPN37 cloned into *Cla*I site of pBluescript AA(−) (Ap)	This study
pJPN45	3.0-kb *Kpn*I-*Xba*I fragment of pJPN31 cloned into the multiple cloning site of pRK415, AA(−) (Tc)	This study

[a] Ap, ampicillin resistance; Tc, tetracycline resistance, Km, kanamycin resistance.

cols on an Applied Biosystems model 380B automated DNA synthesizer.

We have also previously reported the cloning of AAF/I genes into the cosmid vector pCVD301, generating the AA-conferring recombinant cosmid pJPN31 (19). AAF/I regions of this clone were localized by the construction of Tn*phoA* insertion mutants. Detailed mapping of AA(−) insertions suggests that these can be assigned to 10 different specific insertion sites; 2 of these insertions result in alkaline phosphatase expression. Although the full region defined by Tn*phoA* mutagenesis spans approximately 13 kb, insertions mapped to one of two clusters with approximately 9 kb separating the two regions (designated regions 1 and 2 [Fig. 1]). Both alkaline phosphatase-expressing insertions mapped to region 1, with the alkaline phosphatase fusions transcribed from left to right. The *astA-aggA* segment was

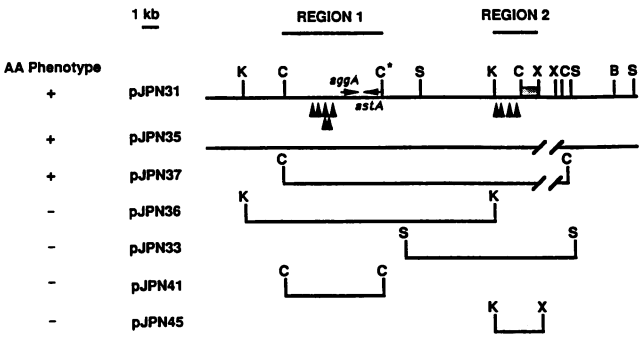


FIG. 1. Map of AAF/I subclones. Various subclones and deletions of cosmid clone pJPN31 were constructed to localize essential AAF/I genes (see text). Region 1 clones pJPN36 and pJPN41 conferred AA only when complemented by either of the region 2 clones pJPN33 (which consists of the 9.4-kb *Sal*I fragment cloned into pACYC177) or pJPN45 (the 3.0-kb *Kpn*I-*Xba*I fragment cloned into pRK415). Restriction sites: C, *Cla*I; S, *Sal*I; K, *Kpn*I; X, *Xba*I; B, *Bam*HI. The *Cla*I site marked with an asterisk represents four *Cla*I recognition sites which are all within a span of 300 bp. Triangles represent sites of Tn*phoA* insertions which inactivate AA. The shaded bar represents the *Xba*I-*Cla*I fragment displaying homology to the oligonucleotide synthesized from the N-terminal amino acid sequence of the 14-kDa protein.

localized at the right end of region 1 by Southern analysis; a *Pst*I-*Eco*RI fragment carrying *aggA* (22) hybridized against the appropriate *Eco*RI, *Sal*I, *Sma*I, and *Nru*I fragments of pJPN31 (data not shown).

AAF/I regions were further localized by constructing various subclones and deletions (Table 1 and Fig. 1). A 1.0-kb *Xba*I fragment was deleted from pJPN31 by digestion with this enzyme and religation of the large fragment. The resulting deletion construction was designated pJPN35; pJPN35 was still capable of conferring AA upon HB101. Tn*phoA* mutagenesis data taken together with data from the *Xba*I deletion experiment suggested that the *Cla*I fragments of 6.8 kb (region 1) and 9.4 kb (region 2) from pJPN31 would contain all the genes necessary to encode AA. Between these two *Cla*I fragments were three small *Cla*I fragments, all totalling less than 300 bp (designated by the asterisk in Fig. 1). pJPN35 plasmid DNA was partially digested with *Cla*I, fragments were ligated to *Cla*I-digested vector pACYC177 (New England Biolabs, Beverly, Mass.), and the reaction product was transformed into HB101. The minimum AA(+) subclone shown in Fig. 1 (designated pJPN37) contained six contiguous *Cla*I fragments: the 6.8- and 9.4-kb fragments, the three small *Cla*I fragments between the two, and a sixth *Cla*I fragment, of 1.6 kb, to the right of the 9.4-kb fragment. The total insert size was 18.1 kb. pJPN37 included all sites implicated by Tn*phoA* mutagenesis. We verified that the configuration of restriction sites in pJPN37 was similar to their organization in the parent strain, 17-2, as well as in pJPN31.

Region 1 was separately cloned as the 6.8-kb *Cla*I fragment into the *Cla*I site of pBluescript II SK (Stratagene Cloning Systems, La Jolla, Calif.). To provide a second selectable marker for region 1, a 2.0-kb cassette bearing *Sal*I ends and encoding kanamycin resistance (derived from pJBK81 [15]) was cloned into the pBluescript II *Sal*I site. This construction was designated pJPN41. Region 2 was separately cloned as the 9.4-kb *Sal*I fragment of pJPN31 into the *Sal*I site of vector pACYC177 (designated pJPN33) and further subcloned as a 3.0-kb *Kpn*I-*Xba*I fragment into the multiple cloning site of pRK415 (designated pJPN45). Both pACYC177 and pRK415 are compatible with pBluescript. When the region 1 clone pJPN41 was transformed into HB101 harboring the region 2 clone pJPN33 or pJPN45, AA

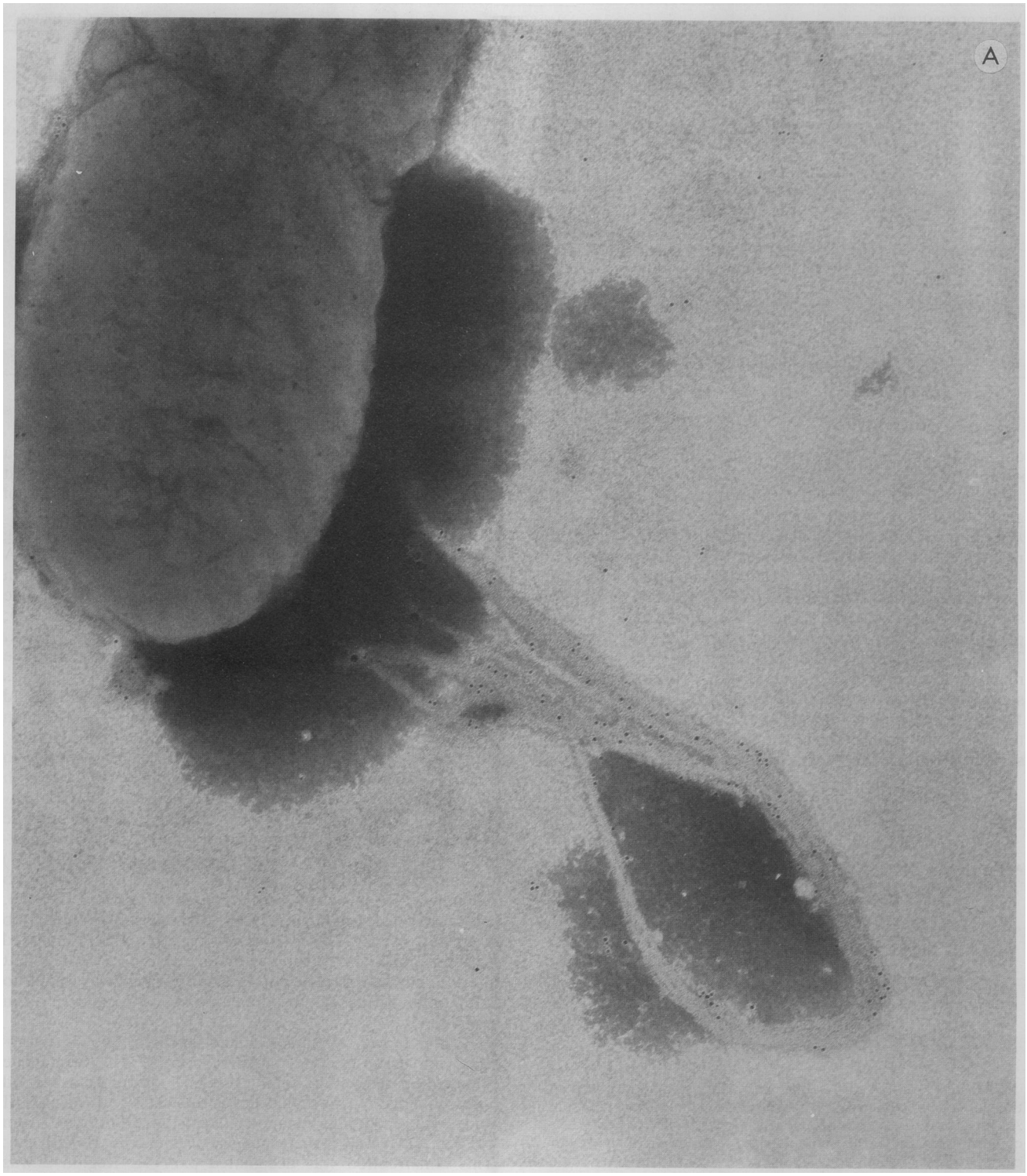
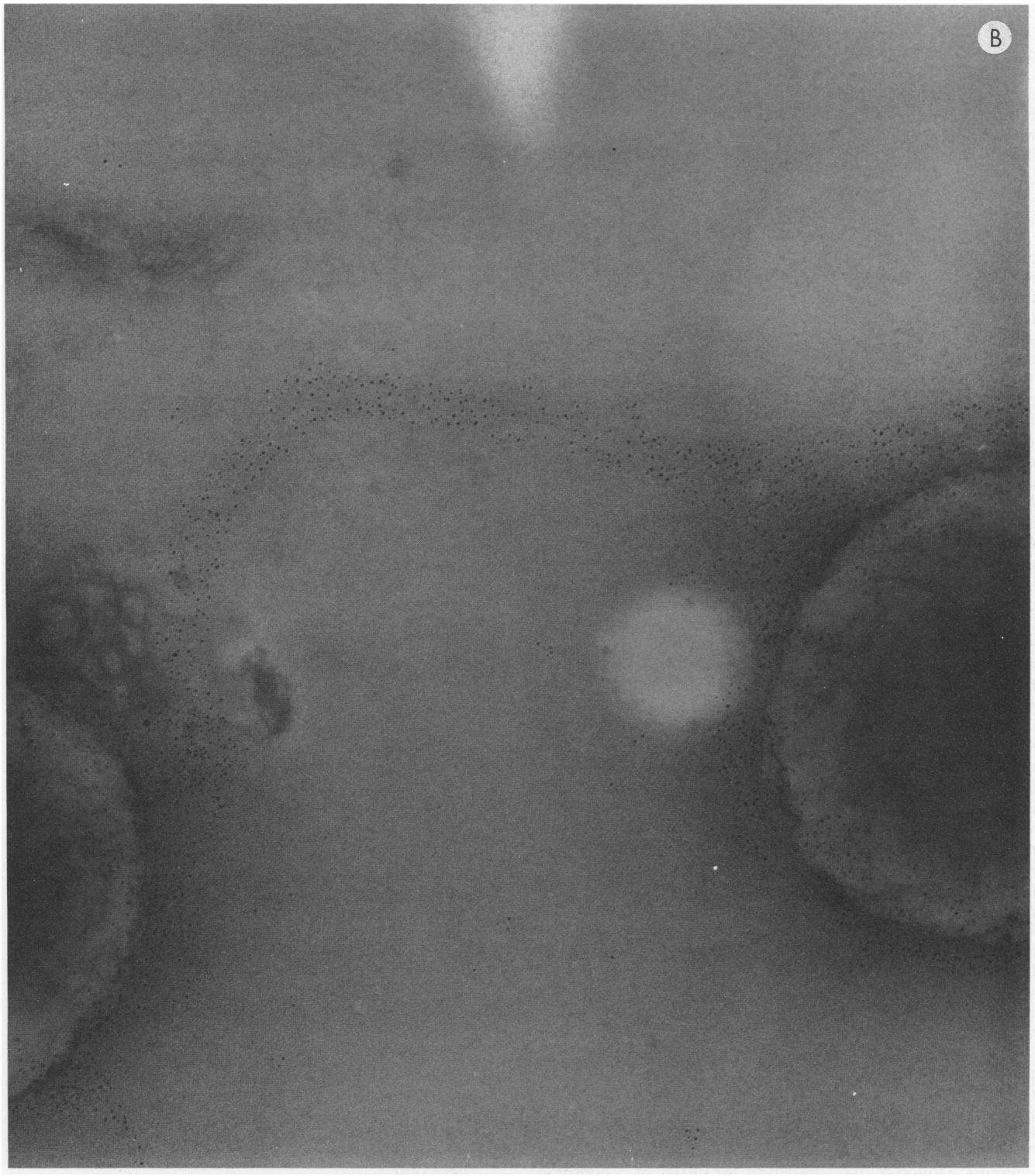


FIG. 2. Immunogold staining for AAF/I. Strains were grown in static L broth overnight at 37°C, and bacteria were pelleted and washed in phosphate-buffered saline. Cells were incubated with absorbed anti-AAF/I antiserum at a 1:400 dilution and then with staphylococcal protein A conjugated to 5-nm-diameter gold particles by standard methods. (A) Parent enteroaggregative *E. coli* 17-2. (B) HB101(pJPN41, pJPN33). Note the typical appearance of AAF/I in bundles which often lie along the surface of the bacterium and connect two cells together. Magnification, ×40,000.



was expressed, whereas pJPN41, pJPN33, and pJPN45 were each incapable of conferring AA individually. The region 1 and 2 clones were also tested for the ability to confer human erythrocyte HA and autoagglutination in L-broth cultures. Like AA, these phenotypes were expressed only when both a region 1 and a region 2 clone were present in the same bacterial cell.

The Tn*phoA* insertion mutagenesis, subcloning, and deletion experiments described above, as well as the localization of *astA*, allowed us to roughly approximate the sizes of

regions 1 and 2. Region 1 is at least 4 kb but no greater than 6.8 kb. The smallest region 2 clone which complemented pJPN41 carries a 3.0-kb insert.

We have previously described the preparation of polyclonal antiserum against AAF/I, which inhibited HA by HB101(pJPN31) (19). By the technique of immunolabelling with colloidal gold, the AAF/I antiserum specifically stained bundle-forming fimbriae on 17-2, HB101(pJPN31), HB101 (pJPN37), HB101(pJPN33, pJPN41), and HB101(pJPN41, pJPN45) but not on HB101(pJPN33) or HB101(pJPN41) (Fig. 2).

Surface shear preparations of HB101(pJPN31) reveal a predominant 14-kDa protein, which we have shown to be recognized by the absorbed AAF/I antiserum on Western immunoblot (19); these properties have suggested this protein to be a candidate fimbrial subunit. The amino acid sequence of the N terminus of the 14-kDa protein was determined to be GGSGW NADNV DPSQX IKLSG VQY. A degenerate oligonucleotide was synthesized according to published codon preferences of *E. coli* (24); the sequence of this oligonucleotide was TGG AAC(T) GCG(C) GAT(C) AAC(T) GTT(G) GAT(C) CCG(A). This oligonucleotide was end labelled with [γ-^{32}P]dATP (New England Nuclear, Beverly, Mass.) using polynucleotide kinase (Promega, Inc., Madison, Wis.). This labelled oligonucleotide was then hybridized by standard methods against Southern blots of *Kpn*I-, *Xba*I-, *Cla*I-, *Sal*I-, and *Sma*I-digested p17-2, pJPN31, pJPN33, pJPN37, and pJPN41. The oligonucleotide hybridized only with plasmids carrying region 2: p17-2, pJPN31, pJPN33, and pJPN37. Oligonucleotide homology localized to a single site on each plasmid, which corresponded to the 1.6-kb *Cla*I fragment of pJPN37, to the left of the unique *Xba*I site (indicated in Fig. 1). The probe did not hybridize with vector pACYC177 or pBluescript II or with pJPN41.

Our data suggest that AAF/I genes are composed of two separate regions of the plasmid which complement each other in trans to encode the fimbriae. We also provide further evidence that the properties of AA to HEp-2 cells, autoagglutination, and human erythrocyte HA all correlate with the presence of the fimbriae demonstrated by immunogold-assisted electron microscopy. The Tn*phoA* data presented here, in combination with the data of Savarino et al. (22), suggest that AAF region 1 has at least two exported peptides: *aggA* and the product(s) identified by our two PhoA-expressing fusions. Region 2 apparently encodes at least the 14-kDa protein, which we have previously shown to be immunogenic in adult volunteers challenged orally with wild-type 17-2 (19). Despite the lack of PhoA-expressing region 2 fusions, the prominence of the 14-kDa protein in shear preparations suggests that it is an exported product.

The position of the EAST1 gene *astA* suggests that the putative virulence loci form a cluster in *E. coli* 17-2. The orientation of PhoA-expressing Tn*phoA* insertions in *aggA* and analysis of *astA* suggest that these two genes are transcribed in opposite orientations (22) (indicated in Fig. 1). This precludes transcription of *aggA* and *astA* from a common promoter, yet regulation by virtue of a common regulatory element is possible.

Most *E. coli* fimbriae are encoded by a cluster of contiguous genes (10). The expression of several fimbriae, however, requires unlinked loci. Many such fimbriae have been shown to be regulated by members of a class of proteins with homology to *rns*, the regulator described for CS1 (5–7, 13, 14). Our sequence analysis of region 2 suggests the presence of an *rns* homolog of 30 kDa (GenBank-EMBL accession no. Z18751) (18). Further experiments, including complete sequencing of both regions, are under way to elucidate the organization and regulation of AAF/I.

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