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A novel dispersin protein in enteroaggregative *Escherichia coli*

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Enteroaggregative *Escherichia coli* (EAEC) is a diarrheal pathogen defined by its characteristic aggregative adherence (AA) to HEp-2 cells in culture. We have previously shown that EAEC strains secrete a 10-kDa protein that is immunogenic in a human EAEC challenge model. We report here that this protein is encoded by a gene (called *aap*) lying immediately upstream of that encoding the AggR transcriptional activator, and that *aap* is under AggR control. The product of *aap* has a typical signal sequence and is secreted to the extracellular milieu, where it remains noncovalently attached to the surface of the bacterium. EAEC *aap* mutants aggregate more intensely than the wild-type parent in a number of assays, forming larger aggregates and fewer individual bacteria. Infection of colonic biopsies with wild-type EAEC strain 042 and its *aap* mutant revealed more dramatic autoagglutination of the mutant compared with the wild-type parent. Our data suggest that the *aap* gene product participates in formation of a surface coat that acts to disperse the bacteria, thus partially counteracting aggregation mediated by aggregative adherence fimbriae. We have therefore named the *aap* gene product “dispersin,” and we propose that it may be representative of a functional class of colonization factors. Since dispersin is expressed *in vivo*, is highly immunogenic, and is present in most EAEC strains, it holds considerable promise as an EAEC immunogen.

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Introduction

Enteroaggregative *Escherichia coli* (EAEC) is an emerging diarrheal pathogen that has been associated with endemic and epidemic diarrheal illness both in developing and industrialized countries (1, 2). The pathogenesis of EAEC infection is thought to comprise adherence to the intestinal mucosa, most likely of both the small and the large intestines, followed by elaboration of one or more enterotoxins (3). These toxins include the heat stable toxin-like EAST1 and plasmid-encoded (Pet) toxin, the latter of which is responsible for mucosal cytotoxicity in explanted colonic

tissue (1, 2, 4–6). Adherence of EAEC to the intestinal mucosa is characterized by the presence of a thick biofilm (2, 7), which may mediate persistence of this organism in the human intestine.

The defining feature of EAEC remains its distinctive aggregative adherence (AA) pattern observed in the HEp-2 adherence assay, in which the bacteria are coinoculated with HEp-2 cells in cell culture medium for 3 hours. In this assay, EAEC adhere to the surface of HEp-2 cells, to the glass substratum, and to each other in a characteristic stacked-brick formation (8). We have shown that AA to HEp-2 cells in well-characterized EAEC strains requires a member of the aggregative adherence fimbriae (AAF) family (9, 10). AAF/I and AAF/II are each encoded on an approximately 100-kb virulence plasmid (designated pAA) (11, 12). The proven human pathogenic strain 042 requires AAF/II fimbriae for adherence to colonic explants (10).

Despite the importance of AAF fimbriae in adherence, we have observed that the majority of EAEC strains lack AAF/I and AAF/II (6). However, our data also suggest that most EAEC strains in our collection carry the pAA plasmid, as evidenced by the presence of several other conserved plasmid loci. Most prominent among these is a transcriptional activator of the AraC

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Nonstandard abbreviations used: enteroaggregative *Escherichia coli* (EAEC); aggregative adherence (AA); aggregative adherence fimbriae (AAF); trichloroacetic acid (TCA).

class, designated AggR, which is required for expression of both AAF/I and AAF/II, but which is also present in a large percentage of EAEC strains that do not express any identified AAF. A second, cryptic locus (originally designated *aspU*) was found in 80% of EAEC strains in our collection (6). Here, we characterize the *aspU* locus, which lies immediately upstream of *aggR* in EAEC 042. This gene encodes a secreted low-molecular weight protein that appears to coat the bacterial surface and thereby promote dispersal of EAEC on the intestinal mucosa. Importantly, the protein, designated dispersin herein, corresponds to a previously cryptic protein against which EAEC-challenged volunteers demonstrated secretory IgA responses (9).

Methods

Cloning and molecular biology methods. Standard molecular biology techniques used published methods (13). *E. coli* DH5 α was used as the host strain for recombinant DNA experiments unless otherwise noted (Table 1).

A mutation in the *aap* gene was constructed using single-crossover insertion mutagenesis as previously described (12). A DNA fragment internal to the *aap* gene was synthesized by PCR, and the fragment was cloned into the *KpnI* and *SacI* sites of suicide vector pJP5603 (14). Primers used to amplify the internal fragment had the following sequence, with restriction sites underlined: 5'-ATGGTACCTTGTTATCTTTCTGGCATCTTGGGT; 5'-ATGAGCTCGAGGGGTAACAACCCCTTGAAGT.

A previously constructed *aggR* mutant strain, JS28, was complemented for AggR expression under control of the *ara* promoter in pBAD30. The *aggR* gene was amplified from 042 by PCR by using the following primers: AggR-forward primer, 5'-GCCCCGGGAT-GAAATTAACAAACATCGAAAA-3'; and AggR-reverse primer, 5'-GCCCCGGGCTATTGGCTTTTAAATAAGT-CAAG-3'. The product was digested with *SmaI* and cloned into the *SmaI* site of expression vector pBAD30.

PCR and RT-PCR for *aap-aggR*. PCR was performed to assess the conservation of the *aap-aggR* locus using the following primers: Aap-forward, 5'-ATGAAAAAAT-TAAGTTTGTATCTT-3'; and AggR-reverse, 5'-GCCCCGGGCTATTGGCTTTTAAATAAGTCAAG-3'.

RT-PCR reactions were performed from Luria broth cultures as previously described (15). Primers used for cDNA synthesis were *cat* (5'-CCACTCATCGCAGTACT-GTT), *aap* (5'-TTATTTAACCCATTCGGTTAGAGC), and *aggR* (5'-GCCCCGGGCTATTGGCTTTTAAATAAGTCAAG). For the PCR step, the reverse primers were as above, paired with the following forward primers: *cat* (5'-TCACTGGATATACCACCGTT), *aap* (5'-ATGAAAAAAT-TAAGTTTGTATCTT), and *aggR* (5'-GCCCCGGGAT-GAAATTAACAAACATCGAAAA).

Expression and purification of the *aap* gene product. To facilitate purification of the *aap* gene product, the *aap* gene was ligated into the *SphI*-*BamHI* sites of plasmid pQE70 downstream of the *lac* promoter. The resulting plasmid, pAap, expresses dispersin fused to six histidine residues at the C-terminus. The *aap* gene was amplified by PCR

using primers with the following sequences: 5'-ACATG-CATGCAAAAAATTAAGTTTGTATC, and 5'-CGGGATC-CAACCCATTCGGTTAGAGC. Amplification results in a DNA fragment with *SphI* and *BamHI* restriction sites at the *aap* upstream and downstream ends, respectively (underlined nucleotides in primers above). The PCR product was digested with *SphI* and *BamHI* and ligated into similarly digested vector pQE70.

The Aap-6His fusion protein was purified from a 100-ml culture volume grown in L-broth with shaking at 37°C to an OD₆₀₀ of 0.6. At this point, IPTG (Sigma Chemical Co., St. Louis, Missouri, USA) was added to a final concentration of 1 mM, and incubation at 37°C was continued for an additional 4 hours. Extraction of the protein was performed according to manufacturer's protocol no. 10 (QIAexpress manual version 3/99; QIAGEN Inc., Valencia, California, USA).

Polyclonal antiserum was raised against the purified Aap-6His protein fusion by subcutaneous injection of rabbits with Aap protein excised from SDS-PAGE gels as described (16). We performed dot blots for AAF/II expression by spotting 100- μ l aliquots from shaking L-broth cultures onto nitrocellulose paper. Blots were then processed using a 1:500 dilution of anti-AAF/II antiserum previously described, and then developed by standard methods using goat anti-rabbit IgG as previously reported (9).

Localization of the *aap* gene product. Overnight L-broth cultures were diluted 1:20 in 5 ml fresh L-broth and grown to log phase. The cells were pelleted for 15 minutes at 1,100 g, and both supernatant and pellet fractions were recovered. The supernatant was precipitated by addition of trichloroacetic acid (TCA) to final concentration of 20%. TCA precipitate was collected by centrifugation at 14,000 g. Loosely adherent surface protein was removed by (a) addition of Triton X-100 detergent (Sigma Chemical Co.) to the L-broth culture (final concentration 0.1%) 1 hour before harvesting of cells; (b) heating of the culture to 60°C for 20 minutes prior to separation of whole cells and supernatant; or (c) vortexing of a 5-ml culture in a VWR Vortex Genie (VWR Scientific, Bridgeport, New Jersey, USA) at the highest setting for 10 minutes.

Adherence and autoagglutination assays. The HEp-2 adherence assay was performed as previously described (17). In certain experiments, purified Aap or BSA (Sigma Chemical Co.) was added to the cells in fresh medium immediately prior to the addition of the bacterial inoculum. Glass-adherence assays were performed as described (15). The autoagglutination assay was performed as described by Hasman et al. (18). Quantitation of adherent organisms was performed by triplicate plate count of serial dilutions removed by 10 minutes' incubation in 1% Triton X-100.

Mucus penetration assay. Mucus penetration was measured by a modification of the method described by Lecker and Sweeney (19). Cells grown overnight in L-broth were diluted 1:200 in L-broth and grown to mid-log phase for 3 hours. Bacteria were pelleted by

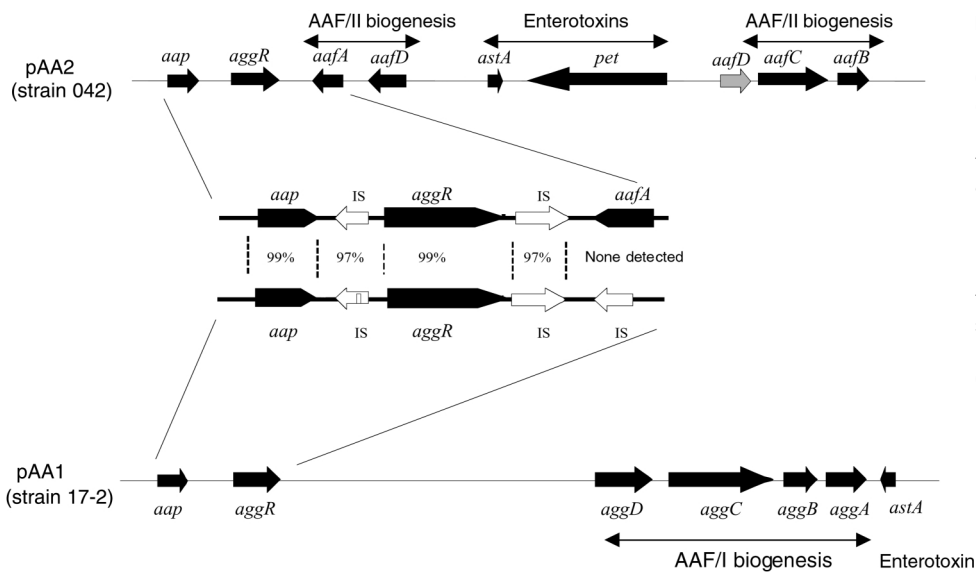


Figure 1 Map of *aap-aggR* loci from strains 042 (producing AAF/II) and 17-2 (producing AAF/I). The *aap-aggR* locus is highly conserved between the two strains, despite divergence of the fimbrial loci. The *aap-aggR* intergenic region is 99% identical in the two strains, except for a 50-nucleotide insertion present in the 17-2 sequence. IS, insertion sequence. Values percent indicate nucleotide homology between 042 and 17-2 sequences.

centrifugation and resuspended in PBS (pH 7.4) to 1×10^7 cells/ml. Bovine submaxillary mucin (Sigma Chemical Co.) was prepared to a concentration of 10 mg/ml in HBSS (GIBCO BRL; Life Technologies Inc, Gaithersburg, Maryland, USA.) and was loaded into a 1-ml tuberculin syringe fitted with a stopcock and 22-gauge needle, creating a mucous column. Ten microliters of cell suspension was gently layered onto the mucin, using a separate column for each strain. The column was incubated at 37°C for 75 minutes in an upright position. Fractions consisting of ten drops each were collected from the bottom of the column. The starting inoculum and each fraction were plated to L agar media with appropriate antibiotics to determine the number of CFUs per ml.

In vitro organ culture. The effect of the *aap* mutation was studied on colonic tissue using pediatric intestinal biopsies in an in vitro organ culture model as previously described (20). Histologically normal samples of transverse colon from six pediatric patients (five male, one female; aged 41–174 months; median age 107 months) were obtained during routine endoscopic examination after ethical approval and informed parental consent.

Electron microscopy. Immunogold transmission electron microscopy for detection of Aap was performed on overnight Luria broth cultures as previously described (10, 21). For scanning electron microscopy, strains were grown in L-broth overnight with shaking at 37°C. Thirty microliters of an overnight bacterial culture was spotted onto silica chips (Ted Pella Inc., Redding, California, USA) previously coated with 0.1% polylysine (Sigma Chemical Co.) and allowed to incubate at room temperature for 5 minutes. The suspension was withdrawn using a Pasteur pipette; then 2% glutaraldehyde (Electron Microscopy Sciences Inc., Fort Washington, Pennsylvania, USA) in CaCo buffer (0.1 M Ca cacodylate, 3 mM CaCl₂; all reagents from Electron Microscopy Sciences Inc.) was applied for 1 hour and incubated at room

temperature. Liquid was again withdrawn, and 0.1 M CaCo buffer was applied three times for 5 minutes per wash. The specimen was then post-fixed with 2% osmium tetroxide (Electron Microscopy Sciences Inc.) in CaCo buffer for 1 hour on ice. The specimens were rinsed twice with distilled water for 5 minutes each. Specimens were then stained with 2% uranyl acetate (Ted Pella Inc.) for 30 minutes at room temperature, rinsed with 50% ethanol, then dehydrated for 5 minutes each in sequential baths of 50%, 70%, 90%, and 100% ethanol. The 100% ethanol wash step was performed three times for 5 minutes each. The specimens were then inserted into a critical point dryer (model CPD-030; BAL-TEC AG, Balzers, Switzerland) until dry, followed by sputter coating in a Desk II Cold Sputtercoater for 90 seconds using platinum and palladium according to the manufacturer's protocols (Denton Vacuum Inc., Moorestown, New Jersey, USA). Specimens were then examined by scanning electron microscopy in a LEO 1550 field emission scanning electron microscope (LEO Electron Microscopy Inc., Thornwood, New York, USA).

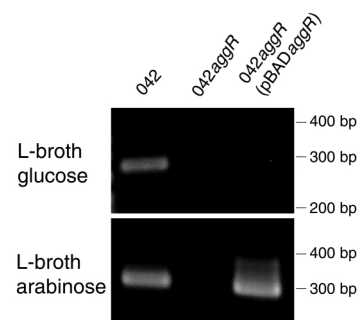


Figure 2 RT-PCR for *aap* transcript in 042, 042*aggR*, and 042*aggR*(pBAD*aggR*). Cultures were grown to late log phase in either L-broth with 0.5% glucose or L-broth with 0.5% arabinose. RT-PCR was performed as described in Methods, and products were separated on 1% agarose gels. The *aap* transcript migrates as a 0.3-kb species as predicted.

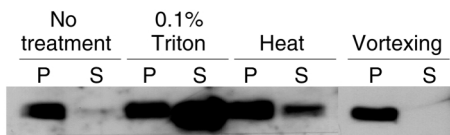


Figure 3
Localization of the *aap* gene product. Western immunoblot analysis was performed on 042 grown in L-broth and subjected to addition of 0.1% Triton X-100 detergent, incubation at 60°C for 20 minutes, or vigorous vortexing for 10 minutes. Cultures were grown to late log phase, and bacteria were pelleted by centrifugation. Pellets (P) were prepared by boiling whole pellet in SDS-PAGE buffer and separating 10% of the sample derived from a 1.0-ml culture. Supernatant (S) was prepared by TCA precipitation. Western immunoblots were performed by standard methods using highly specific polyclonal anti-serum to Aap protein. Aap migrates at the predicted 10-kDa size.

Results

Characterization of the *aap* locus. We have previously reported that *aggR*, encoding a transcriptional activator of AAF expression, is encoded downstream of the fimbrial subunit (*aafA*) on pAA2 in prototype EAEC strain 042 (Figure 1). Sequencing of plasmid DNA upstream of *aggR* revealed an open reading frame (ORF) terminating 815 nucleotides from the *aggR* start codon. The ORF was 348 nucleotides in length, encoding a predicted protein product of 116 amino acids. SignalP analysis strongly predicted a signal sequence with cleavage after position 21. The predicted N-terminal amino acid sequence of the mature species was identical to the N-terminus of a cryptic protein we had previously reported to be secreted from EAEC strain 17-2, and initially surmised to be a fimbrial subunit (ref. 9; GenBank accession no. A49221). The predicted mature protein product of *aap* is 10.2 kDa in size. Neither the nucleotide sequence of *aap* nor its deduced amino acid sequence displays significant identity to any other protein or gene in the databases.

The *aap-aggR* region was remarkably conserved between AAF/I-encoding strain 17-2 (GenBank accession no. Z32523) and AAF/II-encoding strain 042 (Figure 1), despite the fact that the organizations of AAF/I and AAF/II biogenesis genes are dramatically different (12, 22). To assess the conservation of the *aap-aggR* locus among EAEC strains, PCR was performed on total genomic DNA from a selection of EAEC strains previously hybridized with an *aggR* probe (6). Primers were chosen from *aggR* and *aap* in strain 042 such that the PCR product would span the intergenic region. All strains known to hybridize with the *aggR* probe yielded identical 2-kb fragments; all probe-negative strains did not yield a PCR product (not shown).

Since *aap* and *aggR* are separated by less than 1 kb, we asked whether or not the two genes were transcriptionally linked. RT-PCR was performed using the *aap* upstream primer and the *aggR* downstream primer from the experiments above. This RT-PCR reaction did not yield a product, suggesting that the two are not transcribed polycistronically (not shown).

***aap* transcription is *AggR*-dependent.** *AggR* is required for transcriptional activation of AAF/I and AAF/II genes, and its proximity to *aap* suggested that *AggR* might also be required for *aap* expression. To test this hypothesis, we performed RT-PCR for the *aap* transcript in strains 042 and 042*aggR* (Figure 2). A product of the predicted size was seen in 042 but was absent in 042*aggR*. To confirm a requirement for *AggR*, we complemented 042*aggR* with *aggR* cloned under control of the arabinose-dependent promoter in pBAD30. Restoration of *aap* expression was observed in 042(pBAD*aggR*) when cells were grown in the presence of arabinose (*ara*-inducing conditions) but not when they were grown in the presence of glucose (repressing conditions).

Localization of the *aap* gene product. The *aap* gene product has a strongly predicted signal sequence, and previous

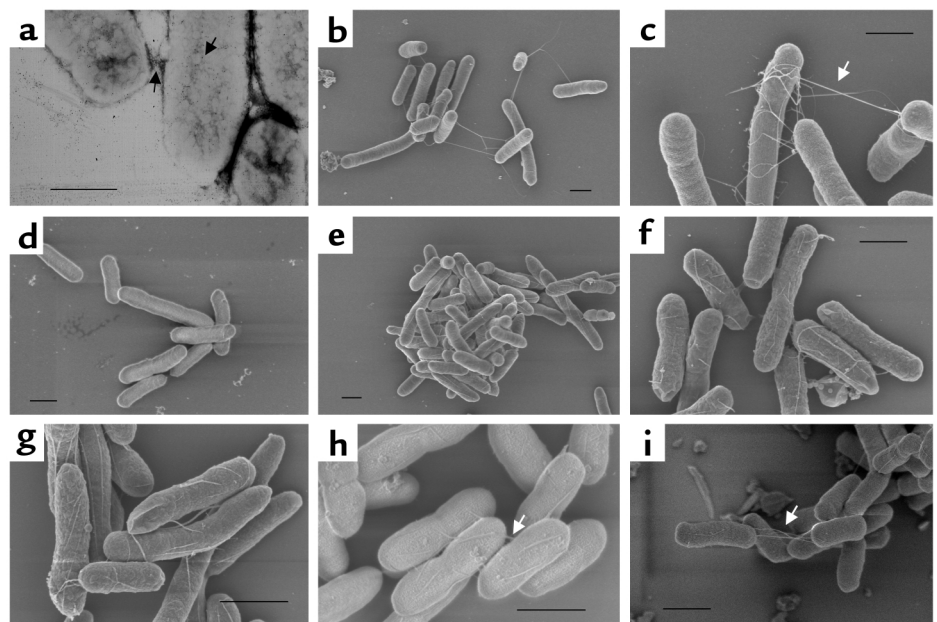


Figure 4
Electron microscopy of 042 and mutants. (a) Immunogold transmission electron microscopy for Aap protein. Black arrows indicate surface protein detected by anti-Aap antibody. Strains examined by scanning electron microscopy are 042 (b and c), 042*aafA* (d), 042*aap* (e-g), and 042*aap*(pAap) (h). In i, 042*aap* was incubated with 3 mg/ml purified Aap protein prior to fixing and staining. White arrows indicate AAF fimbriae in all specimens. All bars indicate 1 μ m.

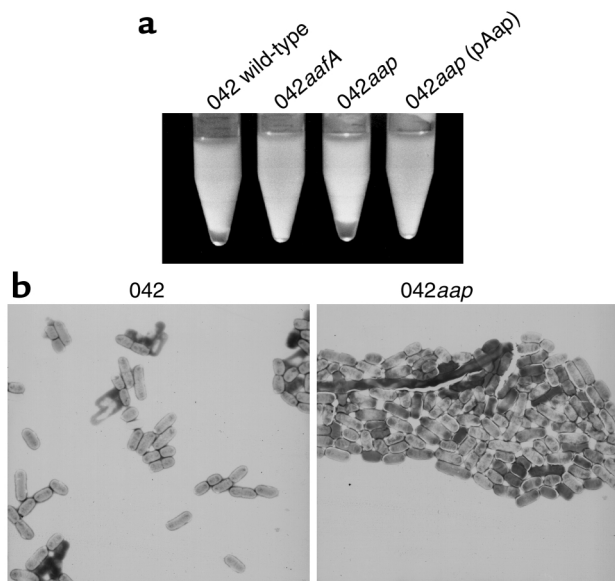


Figure 5
Autoagglutination of 042 and mutants in L-broth. L-broth cultures were grown overnight at 37°C with shaking, then permitted to settle undisturbed at room temperature for 1 hour. (a) Broth cultures revealing a pellet at the bottom of the culture tube correlating with autoagglutination of bacterial cells. (b) Transmission electron microscopy of 042 and 042aap cultures. Ten microliters of L-broth cultures from a were withdrawn prior to incubation on the bench-top and processed for negative staining. Bars, 1 μm.

data have shown that the protein can be found in bacterial supernatants (9). However, quantitative immunoblot experiments suggested that most of the Aap protein pelleted with the bacterium. Experiments were performed to resolve this discrepancy (Figure 3). Broth cultures of 042 were heated to 60°C for 20 minutes or subjected to vigorous vortexing. Both heat and shear forces remove bacterial fimbriae (23). Heat treatment of the culture slightly increased the amount of protein found in the supernatant; vortexing did not release Aap. In contrast, addition of Triton X-100 to a final concentration of 0.1% released the majority of Aap into the supernatant fraction. These data suggest that the *aap* gene product is a peripheral membrane protein, which is noncovalently attached to the bacterial outer membrane.

Electron microscopy of *aap* mutants. The *aap* gene was inactivated in strain 042 by single-crossover homologous recombination of suicide plasmid pJP5603. This construct was complemented by cloning *aap* into plasmid pQE70, under control of the *lac* promoter, and was designated pAap. By immunogold electron microscopy, the surface layer formed a reticulated structure (Figure 4a), which occasionally was visualized between adjacent bacteria (arrows in Figure 4a). 042aap did not stain with the antiserum.

Scanning electron microscopy was also performed on 042 and several mutants (Figure 4, b–i). Under the growth conditions of the assay, 042 expresses only the AAF/II fimbriae, as null mutants in the fimbrial subunit are afimbriate (Figure 4d). AAF/II extends from

the bacterial cell as thin fibers (Figure 4, b and c), joining into thicker bundles and often separating again before attaching the bacterium to the substratum. AAF/II-mediated interbacterial adherence commonly occurred at distances of 2 μm or more (Figure 4c). This appearance was consistent with previous reports (10).

In contrast, 042aap agglutinated more intimately than its wild-type parent, with attachment at a distance seen very rarely (Figure 4, e–g). Interbacterial adherence typically featured bacteria in parallel, with membrane fusions between two bacteria commonly observed (Figure 4f). Moreover, the AAF fimbriae were not observed to extend from the surface of the bacteria but rather lay along the bacterial cell surface, often wrapping around the bacterium (Figure 4, f and g). The complemented *aap* mutant exhibited an intermediate phenotype (Figure 4h), with looser aggregation than that of 042aap. The addition of purified Aap protein to 042aap resulted in partial rescue of the wild-type phenotype (Figure 4i).

Altered adherence and autoagglutination in an *aap* mutant. Because of its location near adherence-related genes in EAEC strains and the appearance of 042aap under scanning electron microscopy, we suspected that the *aap* gene product may play a role in EAEC autoagglutination and adherence. Indeed, stationary-phase broth culture of 042aap exhibited differences in spontaneous settling of bacterial aggregates to the bottom of the culture tube (Figure 5). This difference was reversed in 042aap(pAap) (Figure 5a). Increased aggregation of 042aap was confirmed by transmission electron microscopy (Figure 5b). Aggregation of bacteria in broth cultures was quantitated using the settling assay described by Hasman et al. (18). A typical result of this assay is depicted in Figure 6.

In the conventional 3-hour HEp-2 adherence assay, 042aap displayed dramatically larger aggregates than did its wild-type parent (Figure 7, a and b). This

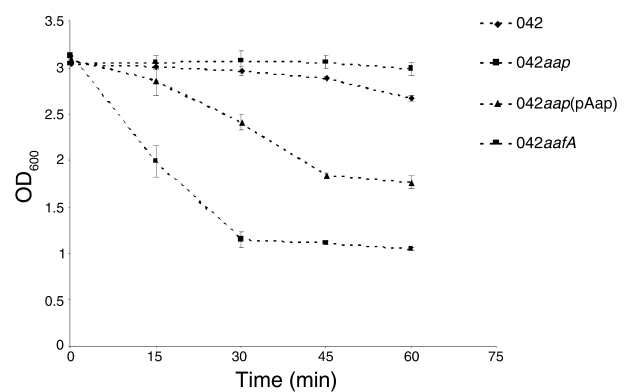


Figure 6
Spontaneous settling of 042 and mutants. Overnight L-broth cultures containing 0.2 mM IPTG were permitted to settle undisturbed at room temperature. At 15-minute intervals, a 100-μl sample was withdrawn from the top of each broth culture and the OD₆₀₀ of the withdrawn sample was determined. Rate of clearing of the uppermost portion of the culture correlates with rate of spontaneous settling, a measure of agglutination. Samples were tested in triplicate. Bars represent SEM.

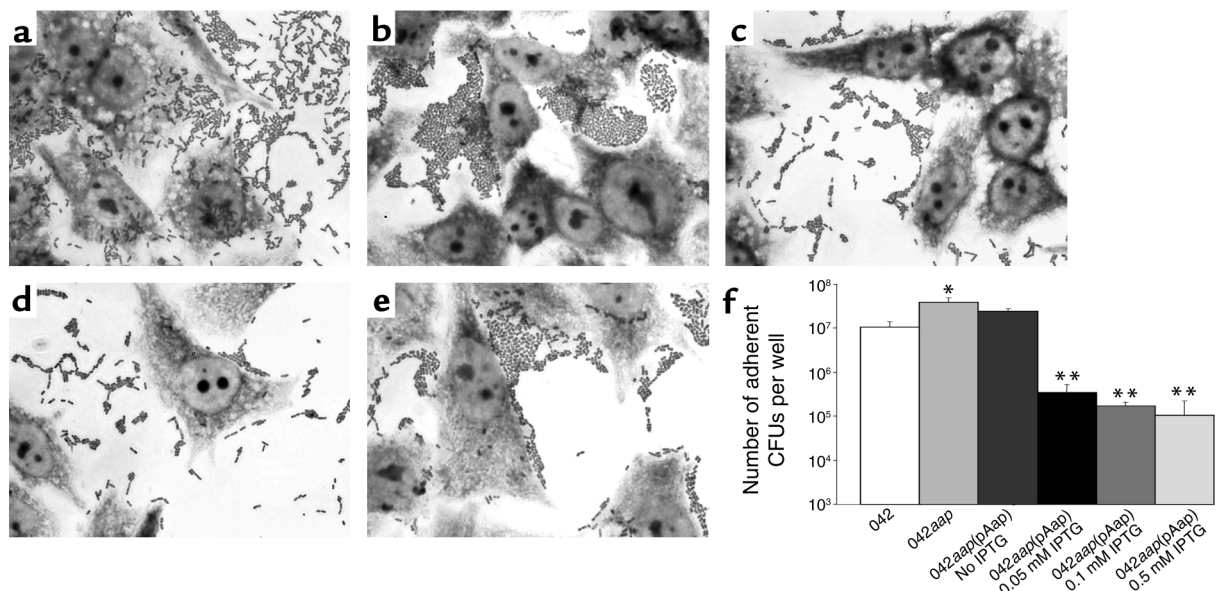


Figure 7

Role of *aap* in epithelial cell adherence. Assays were performed for 3 hours in DMEM–0.5% glucose with 0.2 mM IPTG at 37°C in atmosphere. (a) 042; (b) 042aap; (c) 042aap(pAap). (d and e) The standard HEP-2 cell adherence assay was performed using strain 042aap, but with pure Aap (d) or pure BSA (e) added concurrently with the bacterial inoculum. Each protein was added to a final concentration of 0.1 mg/ml. (f) Quantitation of HEP-2 cell adherence on confluent monolayers growing in DMEM without IPTG (042 and 042aap) or with indicated IPTG concentration [042aap(pAap)]. Bacteria were removed from glass using 1% Triton X-100 and quantitated by viable count. Assays were performed in triplicate, and means are illustrated in histograms with error bars indicating SE. **P* < 0.05 vs. 042 by one-tailed *t* test of log-transformed data; ***P* < 0.05 vs. 042aap by one-tailed *t* test of log-transformed data.

phenotype was also reversed in 042aap(pAap) (Figure 7c). When Aap protein was added to the HEP-2 adherence assay simultaneously with bacterial inoculation, adherence by 042aap returned to levels similar to those seen with wild-type (Figure 7d). Addition of BSA (Figure 7e) with the inoculum or addition of Aap after inoculation (not shown) did not have any effect on adherence. Quantitation of bacterial adherence by plate count revealed a dose-response relationship to IPTG concentration in 042aap(pAap), with two-log reduction in adherence upon addition of 0.05 mM IPTG. This concentration of IPTG does not affect growth rate of the test bacteria (not shown).

When a similar adherence assay was performed in the absence of HEP-2 cells (Figure 8), 042aap produced more abundant adherence to glass than did its parent, whereas adherence of 042aap(pAap) was similar to that of wild-type. Quantitation of this assay (Figure 8d) also revealed a dose-response relationship to IPTG concentration.

Roles for aap in mucosal adherence and mucous layer penetration. We have previously shown that EAEC strain 042 adheres abundantly to the surface of human intestinal biopsy sections in culture (24, 25). To investigate the role of the *aap* gene product in mucosal adherence, 042, 042aap, and 042aap(pAap) were incubated with

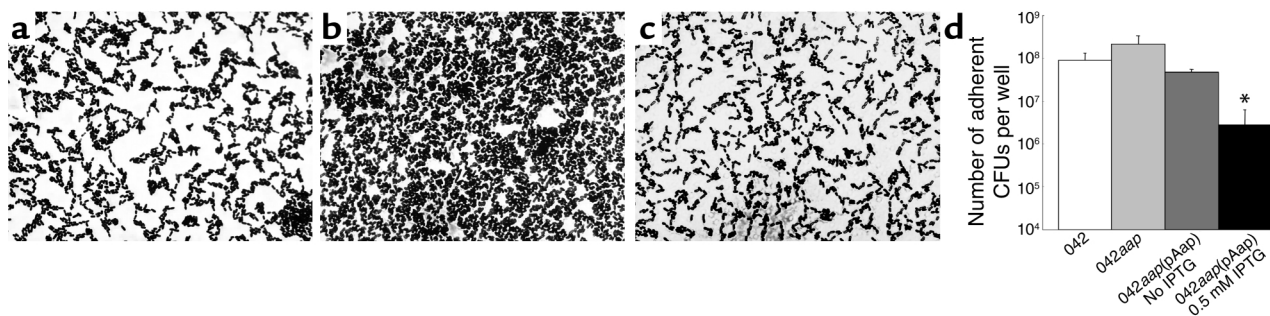


Figure 8

Glass adherence assay. (a–c) Assay was performed for 3 hours in DMEM–0.5% glucose with 0.1 mM IPTG at 37°C in atmosphere. (a) 042; (b) 042aap; (c) 042aap(pAap). (d) Quantitation of adherence grown in DMEM without IPTG (042 and 042aap) or with the indicated concentration of IPTG [(042aap(pAap))]. Bacteria were removed from glass using 1% Triton X-100 and quantitated by viable count. Assays were performed in triplicate, and means are illustrated in histograms with error bars indicating SEM. **P* < 0.05 vs. 042aap by one-tailed *t* test of log-transformed data.

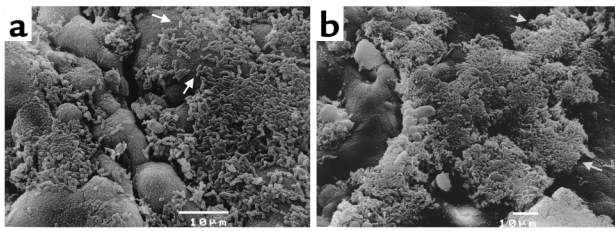


Figure 9
In vitro organ culture assay of 042 (a) and 042aap (b). Bacterial cultures were incubated with colonic biopsies from normal children for 6 hours; then specimens were fixed, stained, and examined by scanning electron microscopy. White arrows (a) denote loose adherence of 042; gray arrows (b) show tight aggregates typical of 042aap. Bars, 10 µm.

fresh colonic biopsy specimens for 6 hours (Figure 9). Wild-type 042 adhered as previously described, producing a biofilm on the absorptive surface of the colonic mucosa, while sparing the colonic glands. Notably, 042 exhibited both aggregates and individual bacteria, very similar to the scanning electron microscopic images from silica chip experiments shown in Figure 4. In contrast, 042aap adhered in larger, tighter aggregates with very few single bacteria observed on the surface of the mucosa.

We hypothesized that decreasing the number of AAF-mediated aggregates could serve to facilitate the penetration of the 042 through the intestinal mucous layer, on the assumption that single bacteria would be more adept at swimming through the mucous gel. To test this hypothesis, we adapted a previously published method (19) in which bacteria are placed at the top of a column composed of purified mucin in water. These experiments revealed that 042aap was more than an order of magnitude less efficient at penetrating the mucin column than was the wild-type parent strain (Figure 10).

Secretion of Aap. Unresolved by the above experiments is the mechanism of Aap translocation past the outer membrane. We observed above that the predicted aap gene product possesses several attributes characteristic of bacterial pilin subunits, including a C-terminal glycine residue that mediates interaction with a PapD-like chaperone protein (26). We have previously shown that AAF/II expression is dependent on the PapD-like chaperone AafD and the PapC usher homolog AafC (12). To assess a possible role for the AafC/AafD secretion system in Aap expression, we assayed Aap in supernatants of 042 with insertional mutations in the aafD or aafC genes. Both mutants secreted Aap (Figure 11). Since aafD and aafC mutants are afimbriate (12), this experiment provides further verification that, though secreted, the Aap protein is not incorporated into the AAF organelle.

Discussion

We have previously shown that most EAEC strains in our collection harbor a partially conserved virulence plasmid (designated pAA), which typically encodes the

transcriptional activator AggR. Here, we show that *aggR* is part of a highly conserved two-gene cluster, which also encodes a novel low-molecular weight secreted protein that mediates dispersal of EAEC on the colonic mucosa. In recognition of this phenotype, we have herein designated this gene *aap* (anti-aggregation protein). We now further suggest the more descriptive name “dispersin” for the *aap* gene product. Our data suggest that dispersin is secreted to the environment, but that it remains noncovalently associated with the bacterial cell surface.

Several roles for dispersin in adherence and colonization are plausible. All enteric pathogens must adhere to the epithelial surface. But at the same time, it may be beneficial to negatively modulate adherence to permit dispersal across the epithelial surface. Knutton et al. have shown that the bundle-forming pilus of enteropathogenic *E. Coli* (EPEC) mediates both aggregation and dispersal, which occur sequentially (27). In this way, EPEC can adhere and multiply early in the pathogenetic sequence, thereby establishing a foothold on the epithelium, but the bacteria then disperse individual progeny, which are free to establish new foci of infection. This paradigm is intuitively beneficial and therefore may be a property of many other mucosal pathogens. Indeed, Benitez et al. have suggested that the Hap mucinase of *Vibrio cholerae* may serve as an enzymatic “detachase,” mutation of which results in increased density of bacterial colonization but also attenuation of virulence (28).

We also hypothesized that dispersin may play a role in penetration of the intestinal mucous blanket, expecting that large aggregates would be retarded in translocation through a viscous gel. Indeed, using a purified mucin gel column assay, we showed that the dispersin mutant penetrated more slowly than the wild-type parent. The human colonic mucosa is covered with a continuous mucous gel approaching 1 mm in thickness

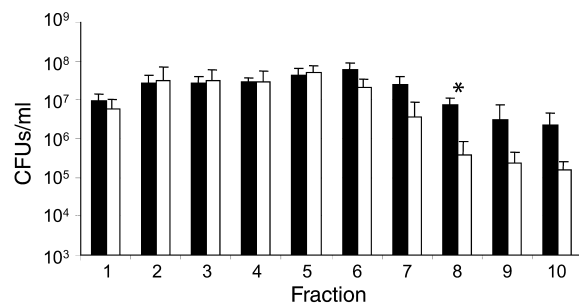


Figure 10
Penetration of 042 and 042aap through mucin. Columns of 10 mg/ml mucin in HBSS were prepared in tuberculin syringes, and the bacterial inocula were applied to the top of the column. The columns were incubated at 37°C for 75 minutes. After this time, the mucin suspension was drained from the column in 0.1-ml fractions, and the number of 042 (black bars) or 042aap (white bars) was determined by quantitative plating in triplicate. Bars represent SD. Fraction 10 represents the bottom of the column, fraction 1 the top. **P* < 0.05 by Student’s *t* test of log-transformed data.

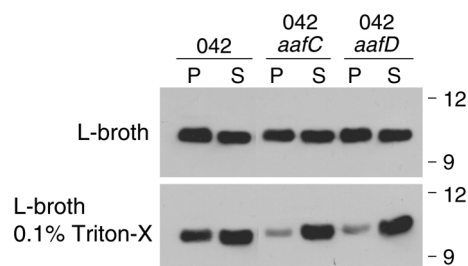


Figure 11

Western immunoblots for Aap performed on supernatants (S) or pellets (P) of overnight L-broth cultures with or without 0.1% Triton X-100. Positions of molecular weight markers are shown at right.

(29), which provides a formidable barrier against colonic pathogens. Single bacteria may be more adept at swimming through this layer; alternatively, biophysical characteristics conferred by the dispersin protein coat could promote mucous layer penetration. Whatever their precise roles, Aap, Hap, and some component of bundle-forming pili are apparently the first examples of bacterial “dispersins,” which may facilitate efficient colonization via bacterial dispersal. Thorough elucidation of the roles of these dispersins *in vivo* has not yet been accomplished, because of the limited availability of good whole-animal models, but is clearly worthy of investigation.

We can propose one potential mechanism by which dispersin may promote dispersal. AAF fimbriae are highly hydrophobic (10), and this hydrophobicity would favor strong autoagglutination in an aqueous environment. The predicted amino sequence of dis-

persin is more hydrophilic than that of AAF/II, and thus the presence of dispersin on the surface of the bacterial cell may ameliorate surface hydrophobicity. Scanning electron microscopy data are consistent with this hypothesis. Absence of dispersin results in striking alteration in the morphology of the AAF fimbriae. In the absence of dispersin, the fimbriae cling to the surface of the bacterial cell, although their number and diameter do not appear changed. This would imply that the surface of the outer membrane under the dispersin coat is largely hydrophobic; such hydrophobicity could be mediated by outer membrane proteins, or perhaps by other hydrophobic surface structures previously described in EAEC (30).

Notably, despite the fact that *aap* is coregulated with AAF adhesins and that dispersin has C-terminal characteristics consistent with secretion by the usher-chaperone pathway, we found that mutants in either the usher or the chaperone secreted normal levels of dispersin. Thus, the mechanism by which dispersin is translocated across the outer membrane is as yet unknown. We found it interesting that AAF mutants bound less dispersin protein in the presence of Triton X-100. Immunofluorescence microscopy experiments suggest that some dispersin is bound within AAF-mediated aggregates (not shown), providing one possible explanation for this observation.

The requirement for AggR in *aap* expression is highly significant. AggR has been shown to be required for expression of AAF biogenesis genes (31), yet there are many strains that carry *aggR* and do not have an identified AAF adhesin (6). Our data thus suggest that AggR acts as a more versatile regulator of virulence

Table 1

Strains and plasmids used in this work

Strains	Characteristics	Reference
042	Wild-type EAEC strain of proven pathogenicity	32
042 <i>aap</i>	042 harboring pJP5603 integrated into the <i>aap</i> gene (Km)	This work
042 <i>aap</i> (pAap)	042 <i>aap</i> carrying the <i>aap</i> gene cloned into vector pQE70 (Km, Ap)	This work
DH5 α (λ <i>pir</i>)	K12 <i>E. coli</i> lysogenized for the <i>pir</i> gene, which permits replication of R6K plasmid replicons	12
S17-1(λ <i>pir</i>)	Conjugative K12 lysogenized for <i>pir</i>	12
042 <i>aafC</i>	042 carrying pJP5603 integrated into the gene encoding the AAF/II fimbrial usher gene (Km)	12
042 <i>aafD</i>	042 carrying pJP5603 integrated into the gene encoding the AAF/II fimbrial chaperone gene (Km)	12
042 <i>aafA</i>	042 carrying <i>TnphaA</i> inserted into the gene encoding the AAF/II fimbrial subunit gene (Km)	10
042 <i>aggR</i>	042 carrying pJP5603 integrated into the gene encoding the regulator AggR (Km)	15
44-1, 6-1, 17-2, 236, 199-1, 103-1, 101-1, 1096, 43996, DS-61R2	Well-characterized, wild-type EAEC strains collected from children in various epidemiologic studies	6
Plasmids		
pQE70	Lac-dependent <i>E. coli</i> expression vector (Ap)	QIAGEN Inc.
pJP5603	R6K suicide plasmid (Km)	14
pAap	<i>aap</i> gene cloned into multiple cloning site of pQE70 to provide IPTG-inducible expression of its gene product as a 6His fusion (Ap)	This work
pBAD30	High-copy number expression vector permitting expression of foreign genes under control of the arabinose operon promoter	33
pBAD <i>aggR</i>	AggR cloned into multiple cloning site of pBAD30 to permit expression of <i>aggR</i> in the presence of arabinose	This work

Ap, ampicillin-resistant; Km, kanamycin-resistant.

genes in EAEC. Indeed, recent data from our laboratory suggest that AggR activates additional unlinked genes on the pAA plasmid and the EAEC chromosome (J. Nishi et al., unpublished results; and E. Dudley et al., unpublished results). Additional characterization of the AggR regulon and its role in EAEC pathogenesis is underway. Coexpression of AAF and dispersin in a common AggR regulon is consistent with an interactive role of the two factors in colonization, but it is interesting that the bacterium would use the same regulator to express both an adhesin and a negative modulator of adhesion.

EAEC is an emerging pathogen, but vaccine development is in its earliest stages. Several obstacles must be overcome before a candidate vaccine can be constructed. Chief among these obstacles is the antigenic heterogeneity of EAEC strains. In addition, given that EAEC infection is a serious health concern predominantly in developing countries, there is little economic incentive toward the development of a vaccine. Therefore, the best approach to EAEC vaccine development would be to identify a highly conserved, highly prevalent, surface-exposed and immunogenic protein that could be expressed in an attenuated vector vaccine. The ideal vector would be one engineered to provide protection against another, perhaps more serious threat (such as shigellosis or typhoid fever). Dispersin fills many of these criteria, and experiments are underway to test its feasibility as an EAEC immunogen.

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