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Article

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Enteroaggregative *Escherichia coli* expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells

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Enteroaggregative *Escherichia coli* (EAEC) is an emerging cause of acute and persistent diarrhea worldwide. EAEC infections are associated with intestinal inflammation and growth impairment in infected children, even in the absence of diarrhea. We previously reported that prototype EAEC strains rapidly induce IL-8 production by Caco-2 intestinal epithelial cells, and that this effect is mediated by a soluble, heat-stable factor released by these bacteria in culture. We herein report the cloning, sequencing, and expression of this biologically active IL-8-releasing factor from EAEC, and its identification as a flagellin that is unique among known expressed proteins. Flagella purified from EAEC 042 and several other EAEC isolates potently release IL-8 from Caco-2 cells; an engineered aflagellar mutant of 042 does not release IL-8. Finally, cloned EAEC flagellin expressed in nonpathogenic *E. coli* as a polyhistidine-tagged fusion protein maintains its proinflammatory activity. These findings demonstrate a major new means by which EAEC may cause intestinal inflammation, persistent diarrhea, and growth impairment that characterize human infection with these organisms. Furthermore, they open new approaches for diagnosis and vaccine development. This novel pathogenic mechanism of EAEC extends an emerging paradigm of bacterial flagella as inflammatory stimuli.

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Introduction

The term enteroaggregative *Escherichia coli* (EAEC) refers to a heterogeneous group of bacteria defined by a phenotypic assay, aggregative (or “stacked-brick”) adherence to HEP-2 cells in culture. Since it was first described in 1987, EAEC has emerged as a leading cause of persistent childhood diarrhea in many developing areas (1, 2). More recently, EAEC has been identified in developed countries, causing acute, persistent, or chronic diarrhea in travelers (3, 4), patients with AIDS (5), and children attending day-care centers (6), and appearing in large food-borne outbreaks (7). In addition to causing diarrhea, pediatric EAEC infections are associated with growth shortfalls and intestinal inflammation, even in children without diarrhea (8). In this setting, we recently documented that early childhood diarrhea correlates with long-term deficits in growth, physical fitness, and cognitive function (9). The long-term effects of EAEC infection on growth and development of children in developing areas may be even more important than the short-term morbidity associated with diarrheal illness.

Based on our clinical studies showing evidence of intestinal inflammation with EAEC infections in children in Brazil, we have reported proinflammatory

activity of EAEC culture supernatants, mediated by a putative protein that causes rapid release of the neutrophil chemokine IL-8 from Caco-2 human colonic carcinoma cells (8). Herein we report the purification, cloning, expression, and identification of this protein as a flagellin unique among known *E. coli* flagellar proteins, but homologous to an unexpressed flagellin from *Shigella dysenteriae*. This finding generates a novel paradigm of *E. coli* enteric pathogenesis.

Methods

Cell culture and IL-8 release. Cell culture reagents were obtained from GIBCO BRL (Bethesda, Maryland, USA) unless otherwise specified. Caco-2 human colonic carcinoma cells were obtained from Cynthia Sears (Johns Hopkins University, Baltimore, Maryland, USA), and were grown as described (8). CCD-18Co human colonic fibroblasts were obtained from American Type Culture Collection (Rockville, Maryland, USA), and were grown in MEM with 2 mM L-glutamine, 1.5 g/L NaHCO₃, 1 mM sodium pyruvate, 0.1 mM nonessential amino acid solution, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 10% FBS. Fibroblasts were serially passaged using 0.25% trypsin and 1 mM EDTA. T84

human colonic carcinoma cells were obtained from American Type Culture Collection and were grown in 50% DMEM and 50% Ham's F12 supplemented with 15 mM HEPES, 26 mM NaHCO₃, 5% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate; these were passaged using 1% trypsin/1 mM EDTA.

For IL-8 release assays, cells seeded at a density of 500,000 per well into 24-well polystyrene plates were used after 5–14 days. Bacteria or bacterial products to be tested were added to 500 µL of growth medium in each well. After 3 hours of incubation at 37°C in an atmosphere of 5% CO₂, the medium was removed and tested for IL-8 concentration by enzyme immunoassay (Quantikine IL-8; R&D Systems, Minneapolis, Minnesota, USA). One unit of activity was defined as the amount of protein that produced half-maximal IL-8 release. When live bacteria were used, the Caco-2 cells were rinsed thoroughly, and the media was replaced with an identical media lacking penicillin and streptomycin before bacteria were added. After 3 hours, supernatants were removed. Cell monolayers were fixed with methanol for 5 minutes and then stained with Giemsa stain for 20 minutes to visualize bacteria and biofilm.

Bacterial preparations. The bacterial isolates used in this study are described in Table 1. For Caco-2 IL-8 release assays, bacteria were grown for 16–24 hours in 1% tryptone at 37°C with shaking at 200–300 rpm. Either 20 µL of live bacterial culture or 50 µL of 0.2 µm-filtered culture supernatant was added to the Caco-2 cells, and the mixture was incubated for 3 hours.

To isolate flagella, bacteria were pelleted by centrifugation, resuspended in 30 mL of 500 mM Tris (pH 8.0), and then blended at high speed for 60 seconds in a Sorvall Omni Mixer (Sorvall Products, Newtown, Connecticut, USA). Bacteria and debris were pelleted by centrifugation at 8,000 g for 15 minutes, and the supernatant was clarified by filtration (0.8 µm pore size). Flagella were pelleted by centrifugation at 100,000 g for 60–90 minutes and then resuspended in 5 mL of PBS. For smaller culture volumes, flagellin was isolated by acid denaturation. Bacterial pellets were suspended in a small volume of 150 mM NaCl and 10 mM HCl, and rotated for 30 minutes at room temperature. Supernatant containing depolymerized flagella was clarified by centrifugation at 100,000 g for 60–90 minutes and equilibrated to pH 8.0 by addition of 50 mM Tris and 10 mM NaOH. The apparent molecular weight of flagellin on SDS-PAGE was the same regardless of the method used.

Protein purification techniques. The IL-8-releasing protein was purified from culture supernatants of EAEC 042, the prototype strain of EAEC that was diarrheagenic in volunteers (10). All purification steps were performed at 4°C. Ultrafiltration membranes and concentrators (Amicon YM-10 membrane, Centricon-30, and Centriplus-30) were obtained from Millipore Corp. (Bedford, Massachusetts, USA). Chromatography was performed using the Gradi-Frac and FPLC systems from Pharmacia Biotech AB (Uppsala, Sweden). The

following chromatographic supports and columns were used: DEAE-cellulose (Sigma Chemical Co., St. Louis, Missouri, USA); Sephacryl S-300 16/60, Mono-Q HR 5/5, Butyl-Sepharose fast flow (Pharmacia Biotech AB); and Bio-Gel HTP hydroxyapatite (Bio-Rad Laboratories Inc., Hercules, California, USA). Protein concentration was measured by the Bradford method (using protein assay reagent from Bio-Rad Laboratories Inc.). Proteins were visualized by electrophoresis through 9% polyacrylamide-SDS gels by the Laemmli method and staining with Coomassie blue, Gelcode Blue (Pierce Chemical Co., Rockford, Illinois, USA), or ammoniacal silver nitrate.

Protein sequencing. After sequential ion exchange, gel permeation, hydrophobic interaction, and hydroxyapatite column chromatography, IL-8-releasing material was concentrated by acetone precipitation and run on a 9% polyacrylamide-SDS gel. The single visible band of approximately 65 kDa was digested in situ with trypsin and sequenced by tandem mass spectrometry at the W.M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia. Homology to published peptide sequences was determined using the BLAST program from the National Center for Biotechnology Information.

Production of aflagellar mutant. The following primers were engineered from the middle third of the *fliC_{SD}* sequence (from nucleotides encoding peptides matching two of those obtained from the EAEC flagellin during mass spectrometry) with *Xba*I and *Eco*RI sites, respectively, at the 5' ends: 5'-TCTAGATAAACTC-GATAATACGGGG-3' and 5'-GAATTCTAACGGCGTTAC-GAAACGTTGT-3'. The 588-bp product amplified with these primers from 042 genomic DNA (using *Taq* polymerase from QIAGEN Inc., Valencia, California, USA) was ligated into the R6K suicide plasmid vector pJP5603, which replicates only when the *pir* gene product is supplied in *trans* (11), and electroporated into DH5α(*λpir*). Transformants were selected by growth on Luria broth (LB) agar with kanamycin (30 µg/mL), and the correct plasmid was identified by restriction pattern. This plasmid was then electroporated into the conjugative strain S17-1(*λpir*), and was conjugated into 042 by filter mating. Transconjugants, predicted to arise by chromosomal insertion of the vector by homologous recombination, were selected by growth on LB with kanamycin (30 µg/mL) and tetracycline (24 µg/mL), and then screened for IL-8-releasing activity. Southern blotting was performed on Zeta-Probe GT membranes (Bio-Rad Laboratories Inc.) according to the manufacturer's protocol. Motility was assessed by growth on swarm plates (1.55 g/L LB and 0.3% agar). HEP-2 adherence was determined as in ref. 2.

Plasmid rescue from transconjugants was performed as follows: Genomic DNA was cut with *Bam*HI and ligated in dilute solution to facilitate homotopic ligation. Ligation product was electroporated into DH5α(*λpir*) and plated on kanamycin agar. Colonies were screened by hybridization with

Table 1

Bacterial isolates used in this study

	Source	Serotype	Mean 3-hour IL-8 release from Caco-2 cells by flagella (pg/mL) ^A	<i>n</i>
EAEC isolates				
042	Peru	O44:H18	564.6	4
1096	Serbia	O4: H?	322.5	2
W452	Thailand ^B	O?:H18	454.6	2
W44	Thailand ^B	O36:H18	< 20	2
W202	Thailand ^B	O?:H18	393.6	2
GDI 44	C	D	< 20	2
GDI 180	C	D	86.5	2
GDI 252	C	D	196.25	1
GDI 299	C	O?:H18	450.6	2
17-2	Chile	ND	30.3	1
Other pathogenic isolates				
<i>V. cholerae</i> (JBK70)	E	-	< 20	2
E2348 (EPEC)	CVD (ref. 30)	O127:H6	283.6	3
933L (EHEC)	F	O157:H7	573.7	2
C1845 (DAEC)	CVD (ref. 30)	ND	< 20	2
Nonpathogenic isolates				
GDI 20	C	D	< 20	3
GDI 16	C	D	190.8	2
BL21(DE3)pLysS	Invitrogen Corp.	ND	< 20	1
Top10F ⁺	Invitrogen Corp.	ND	ND	
DH5 α (λ -pir)	CVD	ND	ND	
S17-1(λ -pir)	CVD	ND	ND	
HB101	CVD	ND	ND	

^AFlagella prepared by acid depolymerization were added to Caco-2 cells at 0.5–5.0 μ g/well and supernatant was assayed for IL-8 by ELISA. ^BObtained from Peter Echeverria (Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand); serotyped by Hermy Lior (Laboratory Centre for Disease Control, Ottawa, Ontario, Canada). ^CGonçaves Dias favela, Fortaleza, Brazil. ^DNot serotyped, but negative for slide agglutination with H18 antiserum. ^E*V. cholerae* expressing only B subunit of cholera toxin; obtained from James Kaper (Center for Vaccine Development, University of Maryland; ref. 29). ^FSLT-I-positive, SLT-II-negative strain, obtained from Alison O'Brien (Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA). ND, not done; EPEC, enteropathogenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; DAEC, diffusely adherent *E. coli*.

the 588-bp probe described above, and the resulting plasmid (~15 kb) was sequenced using the pUC19 forward and reverse primers.

Electron microscopy. Bacteria from saturated overnight culture were pelleted by gentle centrifugation and then resuspended in filtered distilled water. Aliquots (10 μ L) of the suspension were applied to carbon-coated formvar grids for approximately 3–4 minutes. After a quick blotting of the excess suspension, 10 μ L of ammonium molybdate was applied for 5–15 seconds. The stain was then blotted off, and the grids were allowed to air dry. The bacteria were observed and photographed in a JEOL 100CX (JEOL USA Inc., Peabody, Massachusetts, USA) electron microscope at 80 kV.

Cloning and expression of the EAEC flagellin. The following primers were designed from the published NH₂- and COOH-terminal sequences of the *S. dysenteriae* flagellin: 5'-GGATTCATGGACAAGTCATTAAT-3' and 5'-TTCGAATTAACCTGCTGCAGAGA-3'. These were used to amplify a fragment of approximately 1.8 kb from genomic DNA isolated from EAEC 042. This fragment was cloned in frame with a 5' six-histidine (six-His) tag in the pCRT7/NT-TOPO vector (Invitrogen Corp., Carlsbad, California, USA) and maintained in a Top10F⁺ host, according to the manufacturer's protocol. The plasmid containing the *fliC* gene in frame *pfliC* was verified by sequencing, and the fusion protein was expressed in BL21(DE3)pLysS according to the manufacturer's protocol. The control plasmid provided by the manufacturer (pCRT7/NT-E3), expressing a 58-kDa human kinase,

was expressed in parallel as a control. Cells were harvested by centrifugation two hours after induction of mid-log phase culture with 1 mM isopropyl β -D-thiogalactopyranoside (IPTG). Lysis was performed in the manufacturer's recommended lysis buffer by repeated freeze-thaw and sonication. His-tagged proteins were loaded on NiCl₂-charged 1-mL metal chelate columns (Pharmacia Biotech AB) in a buffer containing 500 mM NaCl, 20 mM Tris (pH 8.0), and 5 mM imidazole. They were then washed with 20 mM imidazole and eluted in similar buffer with 100 mM imidazole. The flagellin fusion protein was identified by ELISA using Ni²⁺-conjugated horseradish peroxidase according to the manufacturer's protocol (India HisProbe-HRP; Pierce Chemical Co.) and by Western blot (see below).

Western blotting. Proteins were run on 9% SDS-PAGE gels and electrotransferred to PVDF membranes (Millipore Corp.) at 30 V overnight. They were blocked using 5% nonfat dry milk in TTBS (20 mM Tris at pH 7.5, 150 mM NaCl, and 0.05% Tween-20), and then hybridized for 2 hours with *Salmonella* H antiserum poly a-z (Difco Laboratories, Detroit, Michigan, USA) at a dilution of 1:1,000 in TTBS with 1% gelatin. This was followed by 1 hour with peroxidase-conjugated donkey anti-rabbit IgG (Amersham Life Science, Buckinghamshire, United Kingdom) and developing with ECL light detection reagents (Amersham Life Science). His-tagged proteins were identified by hybridization with India HisProbe-HRP according to the manufacturer's protocol.

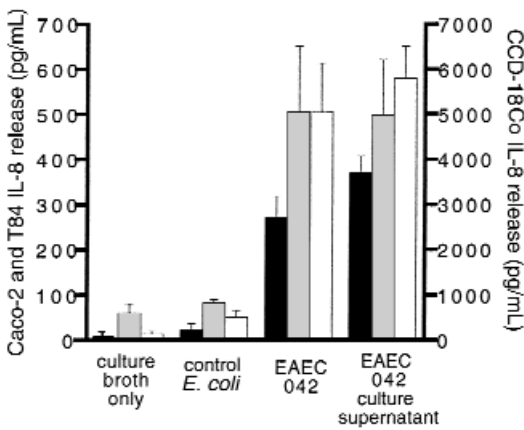


Figure 1

IL-8 release from intestinal cell lines in response to EAEC. Five-day-old monolayers of Caco-2 (black bars), T84 (gray bars), or CCD-18Co (white bars) cells were exposed for 3 hours to live EAEC 042, filtrates of saturated overnight cultures of EAEC 042, nonpathogenic (control) *E. coli* (HB101, K12, or GDI 20), or culture broth alone. The separate Y-axes reflect the tenfold greater amount of IL-8 release by CCD-18Co fibroblasts compared with Caco-2 or T84 colonic carcinoma cells. Error bars represent SEM.

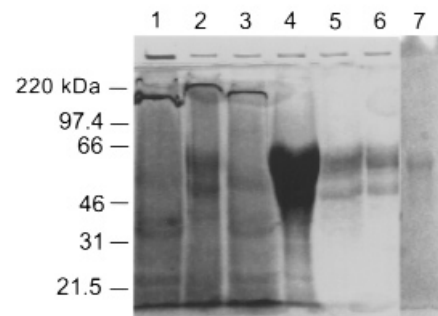
Results

EAEC 042 induces IL-8 release from intestinal cell lines. We previously reported that a prototype EAEC isolate, 042, causes Caco-2 cells to release IL-8 after as little as 3 hours of exposure to either live bacteria or to cell-free culture filtrates. We also demonstrated the heat stability and protease sensitivity of this IL-8-releasing activity, and its lack of inhibition by polymyxin B (8). Because proinflammatory cytokines are regulated differently in various immortalized cell lines, we tested T84 human colonic carcinoma cells and CCD-18Co human colonic fibroblasts. As shown in Figure 1, EAEC 042, the prototype strain that is pathogenic in volunteers, caused significantly more IL-8 release than did nonpathogenic *E. coli* or culture broth alone in all three cell types. Interestingly, T84 and CCD-18Co cells both express measurable IL-8 at baseline, whereas Caco-2 cells do not. For this reason, we used Caco-2 cells as the primary assay cells for the IL-8-releasing protein.

Purification and identification of the IL-8-releasing protein from EAEC 042. We purified the IL-8-releasing protein from culture supernatants of EAEC 042. The appearance of the sequential purification products on 9% SDS-PAGE gels is shown in Figure 2.

Figure 2

Gel (9% SDS-PAGE) of sequentially purified, IL-8-releasing protein fractions from EAEC 042 culture supernatants. Samples were concentrated by membrane ultrafiltration to a volume of 25 μ L and run simultaneously, with the exception of lane 7, which was run in a separate gel. Lane 1: Culture supernatant from overnight growth of 042. Lane 2: DEAE-cellulose batch adsorption and elution with NaCl. Lane 3: Sixty percent ammonium sulfate precipitation. Lane 4: Gel permeation chromatography in the presence of 6 M guanidine-HCl. Lane 5: Pooled, active gel permeation fractions, dialyzed to remove guanidine. Lane 6: Strong anion exchange and subsequent hydrophobic interaction chromatography. Lane 7: Hydroxyapatite chromatography.



The SDS-PAGE band from the hydroxyapatite chromatography step was sequenced by tandem mass spectrometry. Eighteen trypsin peptides were identified that matched predicted sequences from a presumed *S. dysenteriae* flagellin, *fliC_{SD}* (National Center for Biotechnology Information accession 442483). Eleven of these peptides were identical to the corresponding sequences predicted from *fliC_{SD}*; the remainder had single amino-acid substitutions. None of the identified peptides matched predicted peptide sequences of the flagellin from *E. coli* K-12 (*fliC* gene product, accession 120319).

Flagella from EAEC cause IL-8 release from Caco-2 cells. To verify that the identified flagellin was indeed the IL-8-releasing protein from 042, we prepared flagella from EAEC 042 (Figure 3). We cut the higher- and lower-molecular-weight bands from a gel, eluted the proteins into water by diffusion, and dialyzed them thoroughly to remove SDS. The smaller proteins did not cause IL-8 release, whereas the protein of about 65 kDa (henceforth referred to as FliC-EAEC) was extremely potent, with a specific activity of approximately 333 U/ μ g, corresponding to half-maximal activity at approximately 10^{-10} M. Moreover, when EAEC flagella were treated at pH 3.0 and subsequently neutralized, they were still active and maintained their mobility and staining characteristics on SDS-PAGE (not shown). This suggests that depolymerized flagella (consisting largely of the flagellin protein) are as active as intact flagella, and hence that the flagellin itself is responsible for IL-8 release. As with EAEC culture supernatants, addition of polymyxin B (10 μ g/mL) to Caco-2 cells did not reduce the IL-8-releasing effect of EAEC flagellin, suggesting that LPS did not contribute to this effect.

Several additional strains of bacteria were tested for the presence of IL-8-releasing flagella (Table 1). No detectable IL-8 release was induced in Caco-2 cells by the addition of 50 μ L of flagellar solution (0.5–5.0 μ g/well) from either GDI 20 (a nonpathogenic human isolate from Brazil), *Vibrio cholerae*, C1845 (a diffusely adherent *E. coli*), or BL21. In contrast, proinflammatory flagella were detected in eight of ten EAEC isolates from diverse sources, as well as enterohemorrhagic *E. coli* O157:H7 and enteropathogenic *E. coli* E2348. Interestingly, there was a dissociation between reactivity with H18 antiserum and IL-8

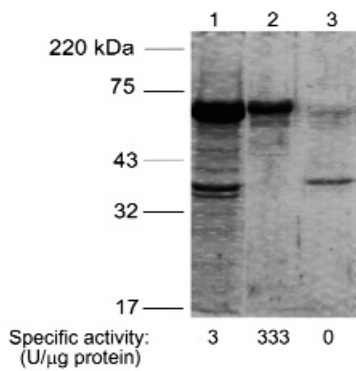


Figure 3

Gel (9% SDS-PAGE) of EAEC flagellar isolation. Samples were concentrated by acetone precipitation before loading. Lane 1: Flagella sheared from EAEC 042 and pelleted by ultracentrifugation. Lane 2: The approximately 65-kDa band, cut and eluted from a previous gel, dialyzed to remove SDS, and reRun in SDS-containing loading buffer. Lane 3: The approximately 35-kDa band similarly eluted, dialyzed, and rerun. Specific activity was defined as the amount of sample producing half-maximal IL-8 release from Caco-2 cells at 3 hours of exposure.

release, suggesting that the H-antigenic epitopes on FliC-EAEC may be separate from the regions of the molecule responsible for proinflammatory activity.

Aflagellar EAEC 042 does not cause IL-8 release. To determine if flagellar expression is necessary for 042 to cause IL-8 release, we used a 588-bp internal fragment of *fliC* to direct insertion of the R6K suicide vector pJP5603 into the EAEC 042 flagellin gene (*fliC*_{EAEC}). This strategy produced a partial merodiploid, 042:*fliC*⁻, harboring the suicide vector (Figure 4a). The identity of 042:*fliC*⁻ as a derivative of 042 was confirmed by antibiotic susceptibility pattern (resistant to chloramphenicol and tetracycline), by aggregative adherence to HEp-2 cells, by biofilm formation, by the presence of a plasmid with the same size and restriction patterns as the 042 AA plasmid, and by agglutination with O44 antiserum (Denka Seiken Co., Tokyo, Japan). Southern hybridization confirmed the site of insertion of the suicide plasmid (Figure 4b). Moreover, plasmid rescue liberated a novel plasmid of approximately 15 kb harboring the R6K origin and kanamycin resistance cassette.

Figure 4

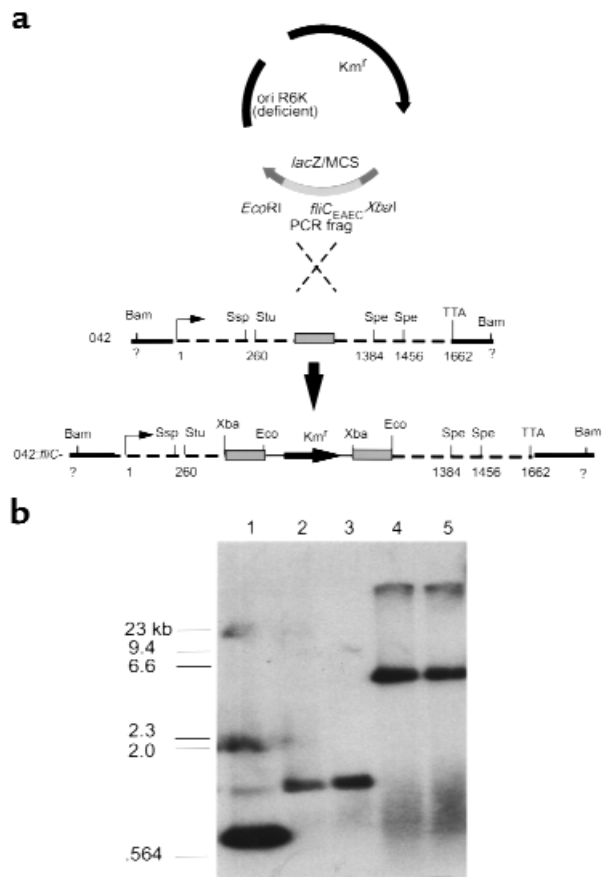
Construction of 042:*fliC*⁻. (a) Restriction maps of pJP5603 (3.1 kb) with the cloned 588-bp PCR fragment of *fliC*_{EAEC} (gray bar), wild-type *fliC*_{EAEC} (dashed line), and the map of the desired transconjugant. Restriction sites and locations are shown (Bam, *Bam*HI; Ssp, *Ssp*I; Stu, *Stu*I; Spe, *Spe*I; Xba, *Xba*I; Eco, *Eco*RI). (b) Southern hybridization of 042 and 042:*fliC*⁻ using the 588-bp PCR fragment from the 042 genome as a probe. Lane 1: Probe, 1 ng. Lane 2: 042, *Spe*I/*Stu*I cut. Lane 3: 042, *Spe*I/*Ssp*I cut. Lane 4: 042:*fliC*⁻, *Spe*I/*Stu*I cut. Lane 5: 042:*fliC*⁻, *Spe*I/*Ssp*I cut. Km^r, kanamycin resistance cassette; TTA, stop codon; ori R6K, origin of replication.

Sequencing of the portions of this plasmid flanking the pJP5603/*fliC* junction revealed the predicted duplicated copy of the 588-bp region of the *fliC* gene, confirming the partial merodiploid (not shown).

IL-8-releasing activities of 042 and 042:*fliC*⁻ were compared using both live bacteria and culture supernatants. As shown in Figure 5c, neither live 042:*fliC*⁻ ($n = 3$) nor its culture supernatants ($n = 5$) caused detectable IL-8 release from Caco-2 cells at similar bacterial density to that from active cultures of 042 (as determined by dilutional colony counts of the bacterial cultures).

042:*fliC*⁻ demonstrated reduced motility compared with wild-type 042 when grown on swarm plates (Figure 5a). Other phenotypic characteristics of 042 (growth, adherence, biofilm formation) were not altered in 042:*fliC*⁻. Reversion to a motile state was able to occur spontaneously in the absence of kanamycin selection, presumably by a recombination event facilitated by the repeated portion of the *fliC* gene. Reversion to wild-type motility also restored IL-8-releasing activity (not shown).

Several strains were examined for flagella by electron microscopy. As shown in Figure 5b, 042 is highly flagellated, whereas 042:*fliC*⁻ has no visible flagella (over many fields). In addition, 042 grown in LB with 3% NaCl had greatly reduced flagellar expression and did not release IL-8 from Caco-2 cells; adding 3% NaCl after overnight growth had no effect on IL-8 release.



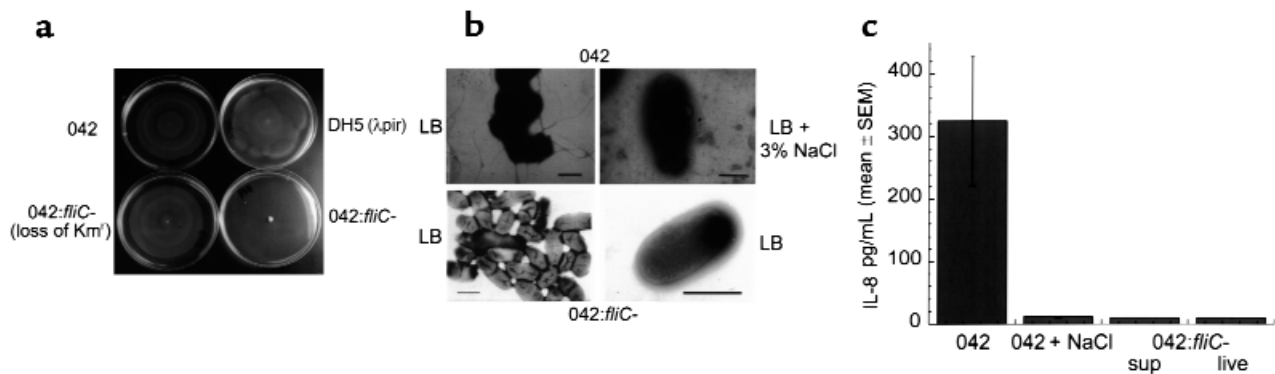


Figure 5

Motility and IL-8-releasing activity of 042 and 042:*fliC*-. (a) Growth at 72 hours on swarm plates without kanamycin. Bacteria picked from the periphery (swarming area) of the lower left plate had lost kanamycin resistance. (b) Electron microscopy with negative staining for flagella. Bar = 1 μ m. (c) IL-8 release from Caco-2 cells incubated for 3 hours with culture supernatants of EAEC 042 grown in 1% tryptone ($n = 5$), 1% tryptone with 3% NaCl ($n = 2$), culture supernatants (sup) ($n = 5$), or live cultures ($n = 3$) of 042:*fliC*- grown in 1% tryptone. $P < 0.05$ by Kruskal-Wallis test.

EAEC flagellin expressed in BL21 causes IL-8 release. As described in Methods, the *fliC* gene was cloned into an expression vector with an NH₂-terminal 6-His tag. Maximal expression occurred 2 hours after addition of IPTG to mid-log phase cultures, although some expression was present in unstimulated cultures, reflecting basal transcription at the T7 promoter. As shown in Figure 6, FliC-EAEC, but not the expressed control protein, immunoreacted with anti-flagellar antibody, whereas both proteins reacted with Ni²⁺-HRP. Crude lysates of BL21-pLysS:*pfliC*, but not BL21-pLysS:pCRT7/NT-E3, caused IL-8 release from Caco-2 cells. The IL-8-releasing specific activity of lysates was increased by purification over a Ni²⁺ column (not shown).

H18 antiserum inhibits IL-8 release by the 042 flagellin. As mentioned earlier, EAEC 042 has a serotype of O44:H18. We used commercially available antiserum to the H18 flagellum (Denka Seiken Co.) to attempt

to adsorb FliC-EAEC and inhibit IL-8 release from Caco-2 cells. This antiserum immunoreacts by Western blot with flagellin purified from 042 or FliC-EAEC expressed in BL21 (not shown). Addition of 1–10 μ L of H18 antiserum to Caco-2 cells inhibited IL-8 release from Caco-2 cells stimulated with 042 culture supernatants, purified flagella, or His-tagged FliC-EAEC to $51.2 \pm 11.2\%$ of control levels (mean \pm SD, $n = 4$, $P = 0.025$). No inhibition of IL-8 release was seen with H7 antiserum or polyclonal anti-*Salmonella a-z* antiserum.

Sequencing of fliC_{EAEC} and comparison to other bacterial flagella. The nucleotide sequence of *fliC_{EAEC}* was obtained from *pfliC* (submitted to GenBank; accession AF 194946). The predicted amino acid sequence of this gene is shown in comparison to flagellins from *S. dysenteriae*, *S. flexneri*, and *E. coli* K12 in Figure 7. The four proteins are nearly identical over the first 150 and last 90 amino acids. FliC-EAEC and FliC-SD are

Figure 6

Expression and purification of FliC-EAEC. (a) BL21(DE3)pLysS transformed with *pfliC* or pCRT7/NT-E3 (expression control) were harvested from mid-log phase cultures at the indicated times after addition of IPTG. Ten microliters of bacterial lysate was loaded per well for 9% SDS-PAGE, and transferred to PVDF membrane after gel was run. The membrane was probed with Ni²⁺-HRP to detect polyhistidine-containing proteins, stripped with 0.2 N NaOH, and reprobed with polyclonal anti-flagellar antiserum. A lane containing 2.5 μ g of EAEC 042 flagella (A) was included in the gel as a control. For IL-8 release, purified flagella (72 ng) or bacterial lysate (1 μ g) was added to 500- μ L wells of Caco-2 cells in the same experiment, and supernatant was assayed after 3 hours of incubation. (b) BL21(DE3)pLysS:*pfliC* was harvested 2 hours after addition of IPTG to mid-log phase culture. Lysates were purified by nickel affinity chromatography, visualized on 9% SDS-PAGE gels, and transferred to a PVDF membrane that was probed with Ni²⁺-HRP. Lane 1: EAEC 042 flagella, 1.4 μ g. Lane 2: Crude lysate, 15 μ g. Lane 3: Flow-through from nickel column, 12.5 μ g. Lane 4: Eluate from column, 10.8 μ g. For IL-8 release, 350 ng of flagella, 1 μ g of bacterial lysate, and 1 μ g of eluted flagellin were tested.

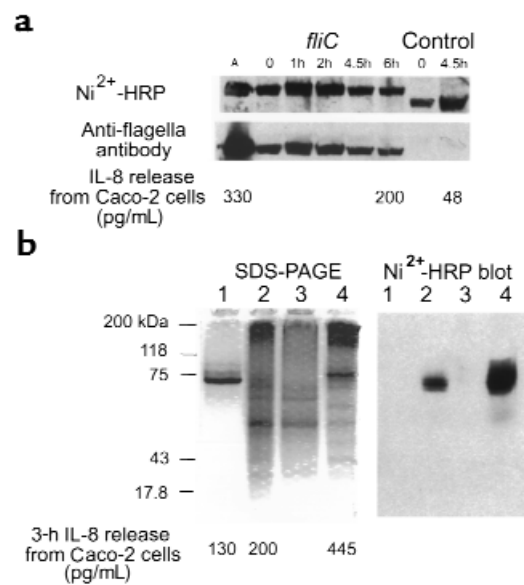


Figure 7

Comparison of deduced amino acid sequences of flagellins from EAEC 042 (EAEC), *S. dysenteriae* (SD), *S. flexneri* (SF), and *E. coli* K12 (K12). Trypsin peptides identified by mass spectrometry from the initial purification product of EAEC 042 supernatants are underlined. Amino acids in the EAEC flagellin differing from those in *S. dysenteriae* are shown above the line. The arrows represent the locations of the forward and reverse primers used to generate the 588-bp *fliC* probe. FliC-EAEC accession AF 194946.

	1		70
EAEC	MAQVINTNSL SLITQNNINK <u>NQALSSSIE</u> RLSSGLRINS AKDDAAGQAI ANRPTSNIKQ LTOAARNAND		
SD	MAQVINTNSL SLITQNNINK NQALSSSIE RLSSGLRINS AKDDAAGQAI ANRPTSNIKQ LTOAARNAND		
SF	MAQVINTNSL SLITQNNINK NQALSSSIE RLSSGLRINS AKDDAAGQAI ANRPTSNIKQ LTOAARNAND		
K12	MAQVINTNSL SLITQNNLNK NQALSSSIE RLSSGLRINS AKDDAAGQAI ANRPTSNIKQ LTOAARNAND		
	71		140
EAEC	<u>GISVAQTTEG</u> <u>ALSEINN</u> NLQ RIRELTVQAT TGTNSDSDL SIQDEIKSRL DEIDRVSGQT QFNGVNVLSK		
SD	GISVAQTTEG ALSEINNQL RIRELTVQAT TGTNSDSDL SI. DEIKSRL DEIDRVSGQT QFNGVNVLSK		
SF	GISVAQTTEG ALSEINNQL RIRELTVQAS TGTNSDSDL SIQDEIKSRL DEIDRVSGQT QFNGVNVLSK		
K12	GISVAQTTEG ALSEINNQL RIRELTVQAT TGTNSDSDL SIQDEIKSRL DEIDRVSGQT QFNGVNVLSK		
	141	⇒	210
EAEC	DGSMKIQVGA NDGETITIDL KKIDS ^D TLNL AGFNVNGEGE TANTAATLKD <u>MVGLKLDNTG</u> <u>VTTAGV</u> NRVI		
SD	DGSMKIQVGA NDGETITIDL KKIDS ^S TLKL TGFNVNGEGE TANTAATLKD MVGLKLDNTG GTTAGVNRVI		
SF	DGSMKIQVGA NDGQTITIDL KKIDS ^D TLGL NGFNVNGGGA VANTAASKAD LVA. . . . AN ATVVG. NKYT		
K12	DGSMKIQVGA NDGETITIDL KKIDS ^D TLGL NGFNVNGKGT ITNKAA ^T VS ^D LTSAGAKLN. . . TPTGL. . . YD		
	211		280
EAEC	. . .ADKAVASS T ^D <u>ILNAV</u> AGV .DGSK ^V STEA D ^V GF ^G ₂ AAPG TPVEy. Ty ^H K DTNTY ^T TASAS VDATQ. . . . L		
SD	. . .ADKAVASS T. IPECDLGV .DGSK ^F TRQM LWRAALVRR I. . . Y. TYIK ILTHHTASAS VDATQ. . . . L		
SF	VSAGYDAAKA SDLL. . .AGV SCD ^G TVQATI NNGFGTAASA TNK ^V YDSASK SYSP ^D TTTAS. . .AAD. . . . V		
K12	LKTENTLLTT DAAFDK. . .L NGDKVTVG. GVDYTYNAKS GDF ^T TTKSTA GTGV ^D AAAQA ADSASKRDL		
	281		350
EAEC	AAFLNPEAGG TTAATVSIG ^N <u>GTTAQEQKVI</u> IAKDGS ^L ATA DDGAALYLD ^D TGNLSK ^T NA ^G TD. TQAKL ^S D		
SD	AAFLNPEAGG TTAATVSIG ^D G ^S IAQEQKVI IAKDGS ^L ATA DDGAALYLD ^D TGNLSK ^T NA ^G TD. TQAKL ^S D		
SF	QKYLTPGVGD TARGTIT ^I . D GSA. . .QDVQ ISSDGKITAS .NGDKLYIDT TGR ^L TKNGSG ASL ^T FEASL ^S T		
K12	AATLHADV ^G K SVNGSY ^T TKD GTVSFETD. . .SAGNIT ^I G. . .GSQAYVD ^D AGNLT ^N NA ^G SAAKADM ^K AL		
	351	←	420
EAEC	<u>LMANNANAKT</u> V. I ^T <u>DKGTF</u> T.ANT TK.FDG VDISVDASTF ^A NAVKNETYT AT ^C GV ^T LPAT		
SD	LMANNANAKT V. I ^P IDKGF T.ANT TK.FDG VDISVDASTF RNAVKNETYT ATVGVHL ^P AT		
SF	LAANTKATT IDIGGTSISF T.GNS TT.PDT ITYSVT ^G AKV DQAADFKA. V STSGMNV ^D FT		
K12	LKAASEGSDG ASL ^T FPNGTEY TIAKATPATT TPVAP ^L IPGG ITYQATVSKD VVLS ^E TKAAA ATSSIT ^F NSG		
	421		490
EAEC	. . .YTVNNGT AA.SA YLVD. .GKVS <u>KT. . .PAEYFA</u> QAD. GTIT. S GENAAT.SKAI		
SD	. . .YTVNNGT AA.SA YLVD. .GKVS KT. . .PAEYFA QAD. GTIT. S GENAAT.SKAI		
SF	TAGYVNGT ^T GAVTKGVDSV YVDN. .NEAL T ^T SDTVDFYL QDD. GSVT. N G.S.GKAV		
K12	VLSK ^T I. GFT AGE ^S S ^D AAKS YVDD ^R G ^G ITN VADYTVSYSV NKDNGSV ^T VA GYASAT ^D TK DYAPAL ^G TAV		
	491		560
EAEC	YVSANGNL ^T T NTTSESEATT NPLAALD ^D AI ASIDKFRSSL <u>GAIQNR</u> LDSA VTNLNNTTTN LSEAQSR ^I QD		
SD	YVSANGNL ^T T NTTSESEATT NPLAALDEAI ASIDKFRSSL GAIQNR ^L DSA VTNLNNTTTN LSEAQSR ^I QD		
SF	YKDADGKL ^T T DAETKAATTA DPLKALDEAI SSIDKFRSSL GAVQNR ^L DSA VTNLNNTTTN LSEAQSR ^I QD		
K12	NVNSAGK ^I TT ETTSAGSATT NPLAALDDAI SSIDKFRSSL GAIQNR ^L DSA VTNLNNTTTN LSEAQSR ^I QD		
	561		600
EAEC	<u>ADYATEVSNM</u> SKAQIIQQAG NSVLAKANQV PQQVLSLQQG		
SD	YDYATEVSNM SKAQIIQQAG NSVLAKANQV PQQVLSLQQG		
SF	ADYATEVSNM SKAQIIQQAG NSVLAKANQV PQQVLSLQQG		
K12	ADYATEVSNM SKAQIIQQAG NSVLAKANQV PQQVLSLQQG		

completely identical over the first 200 residues, and more than 98% identical over the last 420, but only 42% identical from amino acid 220 to 270. A database search of this region of FliC-EAEC using the FASTA program from Genetics Computer Group (Madison, Wisconsin, USA) found no significant homology to any known protein.

Discussion

We have demonstrated that the IL-8-releasing activity of EAEC 042 can be attributed to a single protein moiety that has a sequence very similar to that of a flagellin from *S. dysenteriae*. Furthermore, we have shown that flagella from 042 and several other EAEC isolates are extremely potent in releasing IL-8, and that a flagellar 042 loses its IL-8-releasing activity. Finally, EAEC flagellin expressed as a His-tagged fusion protein possesses the same activity. These findings show that the EAEC IL-8-releasing factor is in fact a flagellar protein that interacts with epithelial cells, leading to IL-8 secretion.

Interestingly, whereas flagella have been implicated in the production of inflammation by several other gram-negative pathogens, this activity has not previously been attributed to a single gene product.

Pseudomonas aeruginosa aflagellar mutants demonstrate reduced pathogenicity in the mouse pneumonia model, and purified flagella can themselves produce pulmonary inflammation (12). In addition, *P. aeruginosa* isolates with altered flagellar expression demonstrate reduced ability to release IL-8 from A549 pulmonary epithelial cells (13). *Helicobacter pylori* isolates with natural polymorphisms in flagellar genes induce different amounts of IL-8 release from MKN45 gastric epithelial cells, and IL-8 release correlates with motility (14). Purified phase I *Salmonella* flagellin and *Salmonella fliC* expressed in *E. coli* each cause release of TNF- α from a human promonocytic cell line (15). Finally, *Salmonella typhi* flagella impair antigen uptake and presentation by human macrophages (16), and induce a cytokine cascade in these cells (17).

Whereas most *E. coli* express flagella and are motile, the closely related *Shigella* species were until recently thought to be devoid of flagella and constitutively non-motile. However, Tominaga et al. demonstrated that *S. flexneri* and *S. sonnei* both possess genes homologous to *fliC* that can be expressed in *E. coli* to produce flagella (18). A corresponding sequence for *S. dysenteriae* was

submitted to GenBank. However, this putative protein has not been described in a publication, nor has any function been ascribed to it.

Flagellar operons are present in all four *Shigella* subgroups, but all contain disrupting elements that prevent coordinated expression of intact flagella (19, 20). Nevertheless, Girón demonstrated that under carefully controlled conditions, *Shigella* can in fact be induced to express flagella, but in very low numbers (one flagellum per 300–1,000 organisms), and that they do exhibit some motility in very soft (0.175%) agar (21). The high degree of homology between the flagellin from EAEC 042 and that from *S. dysenteriae* and *S. flexneri* raises the intriguing hypothesis that expression of flagellin in vivo may contribute to the severe inflammation characteristic of shigellosis.

A comparison of the predicted amino acid sequence of FliC-EAEC with that of other flagellins reveals substantial homology at the NH₂ and COOH termini. This is consistent with the model of flagellin structure proposed by Vonderviszt et al. (22). In monomeric form, flagellin consists of disordered NH₂- and COOH-terminal regions and three organized domains, labeled G1, G2, and G3. The G3 domain, formed by the NH₂- and COOH-subterminal regions of the molecule, forms a compact structure and is highly conserved among many *E. coli* flagellins (23); it presumably is required for proper assembly of the molecule. In contrast, the G1 and G2 domains, which are formed by the middle of the molecule, are hypervariable and presumed to contain the H-antigenic epitopes. The divergence in G1 and G2 domains has been proposed to be driven by antigenic diversity (as a way to avert host response); our work suggests that these domains may confer important and heretofore unrecognized pathogenic phenotypes.

The expression of proinflammatory flagella by EAEC may be an important and novel pathogenic characteristic of these organisms. The phenotype of aggregative adherence, which is pathognomonic of EAEC, is one of several virulence traits identified in these organisms, but is not associated with inflammation. Along with aggregative adherence fimbriae, the large AA plasmid in some isolates carries the genes for Pet, a 104-kDa serine protease enterotoxin and cytotoxin; EAST-1, a small ST-like polypeptide enterotoxin; and several cryptic loci with homology to virulence genes of other bacterial species. None of these genes is required for IL-8 release; plasmid-cured 042 maintains its activity (8). Virulence traits present on the EAEC 042 chromosome include pic mucinase, which is encoded on the antisense strand in an overlapping configuration with *Shigella* enterotoxin 1 (24), and putative genes for yersiniabactin (25) and a putative invasion protein, Tia (26). Although these genes may confer pathogenic phenotypes in vivo, there is no evidence that they are involved in producing inflammation.

Proinflammatory flagella could play an important role in EAEC pathogenesis by inducing epithelial IL-

8 production and recruiting neutrophils to the epithelial surface. In other intestinal infections, neutrophil transmigration has been shown to cause fluid secretion and epithelial barrier disruption (27, 28). This could enhance the effects of other EAEC toxins such as Pet or EAST-1, although whether this occurs in vivo remains to be seen. The presence of IL-8-releasing flagella in diverse EAEC isolates with heterogeneity in other pathogenic genotypes suggests that these flagella may be pathogenic independent of other EAEC toxins. Further studies will be needed to determine the range of effects of proinflammatory flagella on the intestinal epithelium. These findings would in turn lead to a better understanding of how EAEC and other intestinal bacteria produce persistent diarrhea, intestinal inflammation, growth impairment, and other potentially unrecognized manifestations of early childhood infection.

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