Two nonadjacent regions in enteroaggregative *Escherichia coli* flagellin are required for activation of toll-like receptor 5

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Running title: Inflammatory epitopes of EAEC flagellin
Summary

Flagellin is the major structural protein of the flagella of gram-negative bacteria. Recent work has demonstrated that flagellin is a potent trigger of innate immune responses in a number of eukaryotic cells and organisms, including both mammals and plants. In several different human epithelial cell lines, this innate immune response involves toll-like receptor 5 (TLR5). The mechanisms by which flagellin activates TLR5 and the importance of this interaction in other model systems of flagellin-induced inflammation remain unknown. In this work, random and site-directed mutagenesis of the inflammatory flagellin from enteroaggregative *E. coli* (FliC-EAEC) identified two regions in the conserved D1 domain that are required for IL-8 release and TLR5 activation. In contrast, large regions of the variable domain could be excised without reducing the inflammatory activity. In addition, regions of the protein analogous to epitopes which trigger innate immune responses in plants are not involved in Caco-2 flagellin responses. These results highlight the complexity of the interaction between bacterial flagellin and its eukaryotic recognition partners, and provide the basis for further studies to characterize the innate immune response to flagellin.
Introduction

Most gram-negative bacteria express flagella, surface structures that confer motility. Flagella are composed of a basal body that serves as a rotatory motor, a filament that extends into the space around the bacterium to provide motive force, and a hook that connects the two. The filament consists of a long homopolymer of a single protein, flagellin, with a small cap protein at the end. Polymerization of flagellin occurs as a result of relatively conserved structures at the N- and C-termini, although the intervening regions of the protein are highly diverse. The crystal structure of a central proteolytic fragment of flagellin was recently solved, contributing to an understanding of how these conserved structures are involved in filament formation (1).

In addition to their role in bacterial motility, an accumulation of recent evidence suggests that flagella also enhance the pathogenicity of certain organisms, either by promoting adherence to host tissues or by directly activating host inflammatory signalling pathways. Alterations in flagellar expression are associated with decreased virulence in several animal models of bacterial pathogenesis, including *Pseudomonas aeruginosa* lung infection (2-4), *Proteus mirabilis* urinary tract adherence (5), *Helicobacter pylori* gastritis (6), and both nontyphoidal *Salmonella* and *S. typhi* infections (7-12). In addition, bacterial flagella from plant pathogens can induce immune responses in plants such as *Arabidopsis thaliana* and tomato (13-16). These findings suggest that
recognition of bacterial flagella by the innate immune system is a widespread phenomenon among higher eukaryotes.

Recent reports indicate that the component of flagella responsible for eliciting host immune responses is the filament protein, flagellin. Purified or recombinant flagellin causes interleukin-8 (IL-8), nitric oxide, and CCL20 release from Caco-2 intestinal epithelial cells (17-19) and intravenous flagellin causes a systemic inflammatory response in mice (12). *Salmonella typhimurium* flagellin is translocated across T84 cell monolayers during in vitro infection, where it acts basolaterally to cause IL-8 release (20). This effect was shown to be due to activation of toll-like receptor 5 (TLR5) (21). Flagellin from *Listeria monocytogenes* also signals through TLR5, leading to systemic IL-6 release from challenged mice in a MyD88-dependent manner (22).

While it is evident from these reports that flagellin triggers immune responses in several systems, the mechanisms involved and the structural features of flagellin which elicit them remain unknown. In particular, there remains uncertainty as to whether immune activation is specific to flagellins from particular microorganisms, or whether conserved epitopes of flagellin form a pathogen-associated molecular pattern (PAMP) that is recognized by pattern-recognition receptors in a similar fashion to other PAMPs (such as lipopolysaccharide or CpG DNA).

The studies described below were undertaken to answer these questions by examining the inflammatory structural elements of flagellin from enteroaggregative *Escherichia coli* strain 042, a well-characterized diarrheal
pathogen. We have previously shown that this flagellin (termed FliC-EAEC) potently elicits IL-8 secretion from several intestinal cell lines (19). Using both site-directed and random mutagenesis, we demonstrate that disruption of two specific regions in the constant domains of flagellin eliminates IL-8-induction and TLR5 activation.

**Experimental procedures**

*Bacterial isolates.* EAEC strain 042, originally isolated from a child in Chile, was obtained from James Nataro (Center for Vaccine Development, University of Maryland). The cloning and expression hosts Top10F' and BL21 (DE3) pLysS were obtained from Invitrogen (Carlsbad, CA).

*Cloning and mutagenesis.* To express recombinant flagellin, *fliC* genes were amplified from bacterial colonies by PCR, using the following primers: start: 5'-GGATCCATGGCACAAGTCATTAAT-3' and end: 5'-TTCGAATTAACCCTGCTGCAGAGA-3'. The resulting fragments were cloned into pCR7/NT Topo TA (Invitrogen) according to the manufacturer’s instructions. This plasmid contains an N-terminal 6xHis fusion under control of the T7 promoter. This vector containing the *fliC* gene from 042 (p*fliC-EAEC*) was used as the base for subsequent mutagenesis.
Deletion mutants with the exception of Del 0 were generated by two-step PCR and cloned into the BamHI and SfuI sites flanking the fliC gene in pflIC-EAEC. Point mutations were generated by either two-step PCR or by circular PCR with DpnI digestion to eliminate background wild-type plasmid. To create the Del 0 mutant, the BclI-StuI fragment corresponding to bases 34-176 of fliC-EAEC was excised and the gel-purified plasmid backbone blunt-ended with mung bean nuclease (MBI Fermentas, Burlington, ON) to maintain the open reading frame after religation.

Transposon linker mutagenesis was performed on purified pflIC-EAEC using the EZ::TN in-frame linker insertion kit (Epicentre, Madison, WI). Kanamycin-resistant clones were screened for insertion within the fliC gene by restriction digestion. Clones of interest were digested with NotI and religated to yield plasmids with 19 aa insertions in-frame in all six frames. The exact site of the linker insertion was verified by sequencing. Expression of recombinant FliC from these clones was performed as described below; only clones yielding a product of the correct size seen by SDS-PAGE were analyzed further.

**Expression and purification of flagellin.** pflIC-EAEC and its derived vectors with mutant fliC genes were maintained in Top10F'. For expression, plasmids were transformed into BL21(DE3) pLysS and grown in LB with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). Log-phase cultures were induced with 0.5 to 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 37° and then pelleted by centrifugation. Cells were lysed by two freeze-thaw
cycles followed by sonication (three 10 s pulses), and lysates were cleared by centrifugation (15,000 x g for 20 min). Cleared lysates were then tested for Caco-2 cell IL-8 release (2 µl per well; see below). Lysates with absent or reduced activity were purified to verify expression of a full-length protein. These recombinant His-tagged flagellins were purified under native conditions by metal affinity chromatography. Protein expression was verified by electrophoresis on 9% SDS-PAGE, after which proteins were transferred to PVDF membranes and probed with a Ni$^{2+}$-HRP reagent to detect His-tagged proteins (India His-probe HRP, Pierce, Rockford, IL). Eluted proteins in 200-250 mM imidazole were used without buffer exchange to screen for activity on Caco-2 cells, which do not release IL-8 in response to LPS or to imidazole. For experiments on HEp-2 cells, flagellin was first purified of LPS by polymixin B chromatography (Detoxi-Gel, Pierce, Rockford, IL). Flagellin prepared this way had no detectable LPS by Limulus assay (not shown).

Caco-2 IL-8 release. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco’s Modified Eagle Medium with 4.5 g/L D-glucose, 1x sodium pyruvate, 1x nonessential amino acids, 2 mM glutamine, 1x penicillin/streptomycin, and 10% fetal bovine serum. Cell culture reagents and serum were obtained from various sources (Sigma, St. Louis, MO; Gibco, Bethesda, MD; and Hyclone, Logan, UT), and produced no differences in growth or inflammatory response. For IL-8 release assays, cells were seeded into 24-well polystyrene dishes at 5 x 10$^5$/ well and generally used
7-21 days later. To screen flagellin samples for IL-8 release, wells were filled with 500 µl of fresh growth medium and protein samples (up to 10 µg in 50 µl) were added. After 3 h, culture medium was removed and tested for IL-8 concentration by enzyme immunoassay (OptEIA IL-8, BD PharMingen, Mississauga, ON). To determine the EC$_{50}$ of flagellins, three to four five-fold dilutions of flagellin were screened and the inflection point of the dose-response curve was used as the EC$_{50}$.

**Flagellin peptides.** The Flg22 and Flg15$_{EC}$ peptides were provided by Georg Felix (Friedrich Miescher-Institute, Basel, Switzerland). They were synthesized and purified by reverse-phase HPLC. The Flg22 peptide was eluted as a pure single peak and the Flg15 peptide was a partially-purified preparation. Peptides were solubilized at 10 mM or 1 mM in water and diluted in 0.1 M NaCl/0.1 % bovine serum albumin prior to testing.

**TLR5 transient expression.** pEF6/V5-His containing the human TLR5 gene was obtained from A. Aderem (University of Washington, Seattle, WA). pEGFP-N1 (Clontech, Palo Alto, CA) was used as a transfection control. HEp-2 cells were obtained from B. Brett Finlay (University of British Columbia, Vancouver, BC). They were maintained in Ham's F12 medium with 1x penicillin/streptomycin and 5 % fetal bovine serum. Prior to transfection, HEp-2 cells were released with 0.25% trypsin/EDTA and seeded at 10$^5$/ well in 12-well polystyrene dishes. After 24-48 h, the medium was replaced with F12 without
serum. Cells were then transfected with DNA using 0.5 µg of each plasmid and 6.6 µl of ExGen-500 polyethylenimine reagent (MBI Fermentas), per well. Plates were centrifuged for 5 min at 500 x g, incubated for 30 min, and then fed with growth medium. Expression of GFP was confirmed at 24-48 h by fluorescence microscopy. Cells were then fed with 1 ml of fresh growth medium, and flagellin samples added. Supernatants were removed after 3 h for IL-8 ELISA, and cells were rinsed twice with cold Hanks’ Balanced Salt Solution (Sigma) and lysed by freezing/thawing in distilled water. Protein concentration of cell lysates was determined by Bradford Assay (Bio-Rad, Hercules, CA), and fluorescence was measured in a VersaFluor fluorometer (Bio-Rad). Relative fluorescent units were divided by protein concentration to give a measure of transfection efficiency to which IL-8 expression values were standardized.

Circular dichroic spectroscopy. Protein samples were dialyzed against PBS for 24 h and assayed for protein by the Bradford dye-binding method. Samples were diluted to 12.5 µg/ml in PBS and analyzed in triplicate on a Jasco J-810 circular dichroic spectrometer (Jasco, Easton, MD). Tracings were truncated at 200 nm because of the high signal from the buffer below that value. Spectra were subtracted and analyzed using Spectra Manager software (Jasco).

Results.
Two distinct regions of flagellin are required for IL-8-releasing activity.

Based on prior observations showing that only a subset of *E. coli* flagellins were inflammatory (19), we initially hypothesized that the IL-8-releasing activity of EAEC 042 flagellin would be dependent on epitopes in the central, variable domain of the molecule. In order to identify these epitopes, six deletion mutants of flagellin were generated. As shown in Table 1b, only one of these, Del 1, failed to release IL-8 from Caco-2 cells. The region deleted in Del 1 spans amino acids 57 to 190, consisting of the last 121 residues of the N-terminal constant domain and the first 12 residues of the variable domain. None of the remaining sections of the variable domain were required for activity, as evidenced by the wild-type activity of Del 2, Del 3, Del 4, and Del 5 (Table 1a).

In order to screen smaller sections of the protein for activity, random transposon linker mutagenesis was performed on p*flIC*-EAEC. As shown in Figure 1 and Table 1b, four of 37 mutants were devoid of IL-8-releasing activity. Notably, all four of these null mutants were located within a five-residue stretch near the start of the C-terminal constant domain. This finding was of particular interest since several natural polymorphisms which alter flagellar filament structure occur in the corresponding region of *Salmonella typhimurium* FlIC (1). Two of these polymorphisms (G426A and A427V) are analogous to G485 and A486 in FlIC-EAEC, the insertion sites of two of the linker mutations which eliminate IL-8-releasing activity (Figure 1). In order to determine whether the loss of activity could be explained by altered filament structure, several point mutations were generated in this region (Q488A, ΔSLG, and A486L). These
possessed full inflammatory activity, suggesting that localized disruption by the 19-aa linker insertion is likely more important that the alteration or displacement of individual residues, and that altered filament structure cannot fully explain the loss of inflammatory activity.

Characterization of fliC mutants with reduced inflammatory activity.

Several of the transposon mutants obtained were able to elicit IL-8 release from Caco-2 cells, but with reduced potency compared to wild-type FliC-EAEC (Table 1c). These were purified and analyzed by dose-response titration. As shown in Figure 2, most of these mutants (with the exception of 2C3) had the same maximal activity as FliC-EAEC, although they required much higher concentrations to achieve this effect. Mutant 2C3 (linker insertion after E79) had a relatively low EC$_{50}$, but its maximal IL-8 release was less than half that of FliC-EAEC in the same experiment. Interestingly, this insertion is early in the region deleted in mutant Del 1 described above. Only one of the partially-active mutants contained an insertion in the variable domain (after T296). However, this region of the protein is dispensable for IL-8-releasing activity, since Del 3, which lacks this region, is fully active. This suggests that the effects of this linker insertion are most likely due to altered tertiary structure or steric hindrance rather than disruption of a specific epitope.

The least potent of the transposon mutants with reduced activity had its insertion in the disordered region at the C-terminus (after V537). In addition, one engineered mutant with intermediate IL-8-releasing activity, Del 0, lacked most of
the N-terminal disordered region of FliC-EAEC. These regions of the protein were excised prior to crystallization of the *Salmonella* flagellin (1), so their contributions to FliC structure are not certain. However, they are predicted to face the inner tube of the flagellar filament, along with the region disrupted by the noninflammatory transposon mutants (2H3, F8, E11, and C5) (1). This suggests that an interaction between these regions may be required for normal flagellin polymerization as well as for inflammatory activity.

*Regions of flagellin which trigger innate immunity in plant cells are not required for Caco-2 IL-8 release.* It has recently been observed that several plant species exhibit an immune response to flagellins from plant pathogens. A consensus peptide from near the N-terminus of these flagellins (termed Flg22) is a potent stimulant of this inflammatory response, which is mediated by one or more transmembrane proteins with structural similarity to toll-like receptors. FliC-EAEC contains a similar consensus sequence, from E30 through A52. This raised the hypothesis that FliC-EAEC signals inflammation through the corresponding peptide epitope. However, Flg22 and Flg15EC, whose sequences are shown in Table 2, were completely inactive in releasing IL-8 from Caco-2 cells, at concentrations as high as 1 mM. Moreover, they were unable to inhibit IL-8 release due to FliC-EAEC, even at a 10⁵-molar excess. In addition, alterations in this peptide sequence shown to reduce or eliminate signalling in plants were duplicated in FliC-EAEC (Table 2). These mutations did not affect the inflammatory activity of FliC-EAEC (Tables 1a and 2). Finally, the mutant Del
0, described above, maintained partial inflammatory activity despite the complete removal of the region containing the Flg22 sequence. Together, these findings demonstrate that the innate immune response to bacterial flagellin, while present in both mammals and plants, is directed at different epitopes.

*Flagellin mutants devoid of inflammatory activity on Caco-2 cells fail to activate TLR5.* Flagellins from *L. monocytogenes* and *S. typhimurium* activate NF-κB in epithelial cells in a TLR5-dependent manner (21,22). It was previously not known whether FliC-EAEC also activates TLR5, and whether this activation is required for IL-8 release. The most straightforward way to determine this would have been to inhibit TLR5 activity in Caco-2 cells either by transient transfection with a dominant-negative TLR5 construct or with a blocking antibody. However, Caco-2 cells have a poor transfection efficiency, especially after differentiation in confluent monolayers, which is the state at which flagellin IL-8 responses occur. Moreover, no blocking antibody is commercially available. Therefore, HEp-2 cells were used as a model, since they transflect more readily and do not release IL-8 in response to flagellin in their native state.

As shown in Figure 3, HEp-2 cells transfected with a constitutive TLR5 expression vector released significantly more IL-8 in response to flagellin than did untransfected cells. TLR5 expression alone did not lead to IL-8 release. Likewise, the noninflammatory flagellin mutants 2H3 and Del 1 did not cause IL-8 release from HEp-2 cells expressing TLR5. These findings suggest that the same conformational features of flagellin which activate Caco-2 cells are also
required to activate TLR5 in transiently transfected HEp-2 cells, and that the lack of inflammatory activity of the flagellin mutants is likely due to the inability to signal through TLR5.

*The noninflammatory flagellin mutants exhibit circular dichroic spectroscopic profiles similar to wild-type FliC-EAEC.* One possible explanation for the lack of inflammatory activity of Del 1 and 2H3 could be dramatic alterations of protein structure leading to improper folding as opposed to the disruption of a discrete epitope. CD-spectroscopy on FliC-EAEC, 2H3, and Del 1 was used to examine the possibility of gross changes in secondary and tertiary structure. As shown in Figure 4, the spectra of FliC-EAEC and Del 1 were nearly identical, with a shoulder at 220 nm and minimum at 208 nm, consistent with the spectrum of *S. typhimurium* flagellin reported previously (23). The 2H3 mutant had a slight shift of its nadir to 206 nm, but otherwise the tracings were very similar. These findings suggest that the lack of inflammatory activity of these altered flagellins are due to disruption of specific active regions of the protein rather than to major distortions in overall protein structure.

**Discussion.**

The studies reported here demonstrate the complexity of the innate immune recognition of flagellin. The results show that large regions of the variable domain of FliC-EAEC can be deleted or altered by linker insertion without disrupting inflammatory activity. Random linker mutagenesis identified
only one narrow region of the C-terminal constant domain whose alteration
eliminated IL-8 releasing activity. Despite the vulnerability of flagellin to linker
insertions in this site, point mutations there, including some known to alter
filament structure, had no effect on the inflammatory activity. Moreover, deletion
of a length of the N-terminal constant domain (far removed from this site) also
yielded a noninflammatory flagellin. In addition, some linker insertions at a
significant distance from these critical sites attenuated but did not eliminate
inflammatory potency. Finally, the results show that epitopes of flagellin
recognized by innate immune receptors in plants are not required for flagellin
signalling in mammalian cells.

Bacterial flagellins elicit potent inflammatory responses in a number of
eukaryotic cells. In at least two systems, this inflammation is mediated through
TLR5 (21,22). Toll-like receptors are characterized by extracellular leucine-rich
repeats believed to confer ligand specificity, and intracellular Tir domains,
believed to signal via adapter molecules such as MyD88 and IRAK. All of the
TLR activators identified so far are pathogen-associated molecular patterns
(PAMPs), molecular signatures found in microorganisms but not in eukaryotic
hosts (such as LPS, lipoteichoic acid, dsRNA, and unmethylated CpG DNA). If
TLR5 signalling is analogous, then flagellin should display a PAMP which
eukaryotic organisms have learned to recognize through co-evolution with
bacteria. For this hypothesis to be correct, the TLR5 recognition site(s) of
flagellin would have to be located in the conserved domains of the protein, since
the variable domains are highly divergent. This would, in turn, suggest that
flagellin from most, if not all, enteric gram-negative bacteria would be able to activate TLR5, since they share these conserved domains. Some evidence in support of this hypothesis is that culture filtrates from nonpathogenic, gut-derived, flagellated *E. coli* were able to induce IL-8 release from T-84 cells and NO release from DLD-1 cells (12,21). However, there are other reports of nonpathogenic *E. coli* strains failing to induce the CCL20 chemokine (18), to activate TLR5 (22), or to elicit IL-8 release from epithelial cells (19). Thus, the question of whether inflammatory activity is a widespread feature of gram-negative flagellins or a specific trait of individual pathogens remains unanswered.

One way to address these questions is to generate flagellin mutants and to screen them for activity. There are two reports of such experiments, with conflicting results. McDermott et al (24) found that TNF-α release from monocytes was induced by full-length *Salmonella enteritidis* flagellin or by a peptide spanning the variable domain (although at reduced potency). Deletion of either the N- or C-terminal half of the variable domain reduced the potency further but still yielded an inflammatory molecule. However, a flagellin completely lacking the variable domain was inactive. These findings led to the conclusion that the variable domain of flagellin contains two separate inflammatory epitopes, but that tertiary structure conferred by the constant domains is required to display these epitopes effectively. In contrast to these findings, Eaves-Pyles et al (17) showed that a construct containing the N- and C-terminal constant regions of *Salmonella dublin* flagellin separated by an *E. coli* hinge element was as active as wild-type flagellin. They concluded from these
data that the constant domains of flagellin contain the inflammatory epitopes, and that the variable domains are necessary only to keep the constant regions in proper apposition.

There are several characteristics of flagellin which make these questions difficult to answer conclusively. One is that the protein autopolymerizes at high concentrations or in the presence of certain salts (25), making crystalization of the full-length protein elusive so far. The fact that recombinant flagellin is as active as flagellar filaments sheared from live bacteria suggests that full-length filament formation is not required for inflammatory activity. However, it is not known whether some degree of autopolymerization of recombinant molecules occurs under the conditions tested in vitro. In addition, certain pathogenic bacteria can post-translationally modify flagellin, and the importance of these modified residues to inflammation has not been examined (11).

The findings reported here substantially increase knowledge regarding the molecular basis for innate immune recognition of flagellin. We demonstrate that linker insertions within a 5-residue stretch of the C-terminal constant domain of FLIC-EAEC eliminated both Caco-2 cell IL-8 release and TLR5-mediated responses in HEp-2 cells. Moreover, deletion of portions of the N-terminal constant region also abrogated the inflammatory response to flagellin. In contrast, most of the variable domain appeared to be dispensible for this activity.

The presumed locations of these mutations on the folded flagellin protein (based on the crystal structure of the S. typhimurium fragment (1)) are shown in Figure 5. The transposon linker insertions which eliminate inflammatory activity
(2H3, E8, F11, C5) are located near the midpoint of the α-helix formed by the C-terminal conserved domain of the protein. The Del 1 mutation removes the apposed α-helix formed by the N-terminal conserved domain. These regions are predicted by the crystal analysis to form the axial skeleton of the flagellar filament. The region altered by the transposon insertions is predicted to face the inner hollow tube of the filament, along with portions of the extreme N- and C-termini which are believed to form loose, unstructured helices but were excised prior to crystallization (1,23). This same region of the protein is the site of several natural polymorphisms in the *Salmonella* flagellin which confer altered filament structure (1). Together, these findings suggest that innate immune recognition of flagellin requires a tertiary structural element formed by the apposition of the two long α-helices within the D1 domain. The partial reduction in inflammatory activity of mutants Del 0, 2C3, and 2D12 could be explained by disruption of the distal portions of these helices, leading to altered tertiary structure.

This model does not explain the reduced inflammatory activity of mutant E9, which carries its insertion in the D3 domain, far removed from the presumed critical site. Nor does the model explain why various groups have reported that some flagellins expressed by nonpathogenic *E. coli* are noninflammatory, since they presumably must share these critical conserved structures in order to make functional flagella. It is possible that these noninflammatory flagellins carry one or more spontaneous point mutations or deletions within the D1 region. Alternatively, there might be post-translational modifications of critical residues
within the D1 domain which affect innate immune recognition but not filament formation. Further studies to address these questions should shed considerable light on how flagellin is recognized by mammalian cells.

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1. Samatey, F. A., Imada, K., Nagashima, S., Vonderviszt, F., Kumasaka, T., Yamamoto, M., and Namba, K. (2001) *Nature* **410**, 331-337

2. DiMango, E., Zar, H. J., Bryan, R., and Prince, A. (1995) *J Clin Invest* **96**, 2204-2210

3. Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H., and Prince, A. (1998) *Infect Immun* **66**, 43-51

4. Ramphal, R., Arora, S. K., and Ritchings, B. W. (1996) *Am J Resp Crit Care Med* **154**, S170-174

5. Mobley, H. L., Belas, R., Lockatell, V., Chippendale, G., Trifillis, A. L., Johnson, D. E., and Warren, J. W. (1996) *Infect Immun* **64**, 5332-5340
6. Ohta-Tada, U., Takagi, A., Koga, Y., Kamiya, S., and Miwa, T. (1997) *Scand J Gastroenterol* 32, 455-459

7. Wyant, T. L., Tanner, M. K., and Sztein, M. B. (1999) *Infect Immun* 67, 3619-3624

8. Wyant, T. L., Tanner, M. K., and Sztein, M. B. (1999) *Infect Immun* 67, 1338-1346

9. Ikeda, J. S., Schmitt, C. K., Weinstein, D. L., Metcalf, E. S., and O’Brien, A. D. (2000) in *100th General Meeting of the American Society for Microbiology*, p. 81, American Society for Microbiology, Los Angeles.

10. Igimi, S., Amano, F., and Kumagai, S. (2000) in *100th General Meeting of the American Society for Microbiology*, p. 62, American Society for Microbiology, Los Angeles.

11. Ciacci-Woolwine, F., Blomfield, I. C., Richardson, S. H., and Mizel, S. B. (1998) *Infect Immun* 66, 1127-1134

12. Eaves-Pyles, T., Murthy, K., Liaudet, L., Virag, L., Ross, G., Soriano, F. G., Szabo, C., and Salzman, A. L. (2001) *J Immunol* 166, 1248-1260

13. Felix, G., Duran, J. D., Volko, S., and Boller, T. (1999) *Plant J* 18, 265-276

14. Bauer, Z., Gomez-Gomez, L., Boller, T., and Felix, G. (2001) *J Biol Chem* 276, 45669-45676

15. Meindl, T., Boller, T., and Felix, G. (2000) *Plant Cell* 12, 1783-1794

16. Gomez-Gomez, L., Felix, G., and Boller, T. (1999) *Plant J* 18, 277-284

17. Eaves-Pyles, T. D., Wong, H. R., Odoms, K., and Pyles, R. B. (2001) *J Immunol* 167, 7009-7016
18. Sierro, F., Dubois, B., Coste, A., Kaiserlian, D., Kraehenbuhl, J. P., and Sirard, J. C. (2001) Proc Natl Acad Sci U S A 98, 13722-13727

19. Steiner, T., Nataro, J., Poteet-Smith, C., Smith, J., and Guerrant, R. (2000) J Clin Invest 105

20. Gewirtz, A. T., Simon, P., Jr., Schmitt, C. K., Taylor, L. J., Hagedorn, C. H., O’Brien, A. D., Neish, A. S., and Madara, J. L. (2001) J Clin Invest 107, 99-109

21. Gewirtz, A. T., Navas, T. A., Lyons, S., Godowski, P. J., and Madara, J. L. (2001) J Immunol 167, 1882-1885

22. Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., and Aderem, A. (2001) Nature 410, 1099-1103

23. Steiner, T., Lima, A., Nataro, J., and Guerrant, R. (1998) JID 177, 88-96

24. Vonderviszt, F., Kanto, S., Aizawa, S., and Namba, K. (1989) J Mol Biol 209, 127-133

25. McDermott, P. F., Ciacci-Woolwine, F., Snipes, J. A., and Mizel, S. B. (2000) Infect Immun 68, 5525-5529

26. Samatey, F. A., Imada, K., Vonderviszt, F., Shirakihara, Y., and Namba, K. (2000) J Struct Biol 132, 106-111

Footnotes
1. Abbreviations used in the text: TLR5--Toll-like receptor 5. EAEC--enteroaggregative Escherichia coli. IL-8--Interleukin-8. MyD88--Myeloid differentiation factor 88. PAMP--pathogen-associated molecular pattern. FliC--flagellin. aa--amino acid. LB--Luria broth. IPTG--isopropyl-ß-D-thiogalactopyranoside. PVDF--polyvinylidene difluoride. LPS--lipopolysaccharide. HRP--horseradish peroxidase. EIA--enzyme immunoassay. EDTA--ethylenediaminetetraacetic acid. GFP--green fluorescent protein. PBS--phosphate-buffered saline. NO--nitric oxide. TNF-α--tumor necrosis factor-α.

**Figure legends**

Figure 1: Amino acid sequence of FliC-EAEC, showing sites of transposon linker insertions which preserve (arrowheads), reduce (triangles), or eliminate (stars) IL-8-releasing activity on Caco-2 cells. The constant domains of the protein are underlined.

Figure 2: IL-8 secretion from Caco-2 cells treated with increasing doses of wild-type FliC-EAEC or various altered flagellins. Each dose-response curve was generated from a single experiment along with FliC-EAEC and IL-8 values were expressed as the percentage of maximal IL-8 release in the same experiment. The maximal amounts of IL-8 release ranged from 500 to 2000 pg/ml, depending on the age of the Caco-2 cells in culture. Results are representative of at least 2 replicates.

Figure 3: Noninflammatory flagellin mutants fail to cause TLR5-mediated IL-8 release. HEp-2 cells were transfected with pEGFP alone (“untransfected”) or pEGFP plus pEF6-V5-His-TLR5. After 24-48 h, cells were exposed to 1 µg of the flagellins shown or to PBS control for 3 hours. IL-8 expression ratios were determined as described in Experimental Procedures. Replicate numbers are as shown. The amount of IL-8 released by FliC-EAEC in TLR5-transfected HEp-2 cells in individual experiments ranged from 163 to 888 pg/ml, depending on the transfection efficiency and cell number.
Figure 4: CD spectroscopy of flagellins. FliC-EAEC (solid line), Del 1 (dotted line), and 2H3 (dashed line) flagellins were diluted to 12.5 µg/ml in PBS and analyzed for far-UV circular dichroism. The tracing of PBS alone was subtracted from each result. (A) raw data (B) normalized data.

Figure 5: Schematic diagram of the predicted structure of FliC-EAEC, its position in the flagellar protofilament, and the locations of mutations described in this paper. D1 refers to the conserved domain of largely α-helices, (from approx. N57 to G177 and E461 to Q509). The terminal disordered regions are represented as coiled lines, with the N- and C-termini as indicated. D2 and D3 are the two predicted globular domains formed by the variable region (G177 to E461). The borders of the regions removed in Del 1 and Del 0 are indicated by dark lines. These structures are predicted based on crystal analysis of the Salmonella typhimurium flagellin and areas of sequence homology (1). The depiction of the variable domains, in particular, may differ from this representation.
### Tables

Table 1a: Engineered mutants of FliC-EAEC with preserved IL-8-releasing activity on Caco-2 cells

| Name   | Description                        |
|--------|-----------------------------------|
| G47A   | G47A                              |
| Atum   | I38V, N39G, S40Q                  |
| ΔQA    | Q48L, A49Q                        |
| Q488A  | Q488A                             |
| A486L  | A486L                             |
| ΔSLG   | S483A, L484A, G485A               |
| Δ5     | N404V                             |
| Δ2     | I433A, T434A, S435A, G436A        |
| del 2  | T187-S266                         |
| del 3  | V267-Q336                         |
| del 4  | A337-T394                         |
| del 5  | L395-T441                         |
Table 1b: Mutants with absent IL-8-releasing activity (at doses up to 10 µg)

| Sample | Type of mutant       | Site of mutation |
|--------|----------------------|------------------|
| del 1  | engineered deletion  | N57-D190         |
| 2H3    | transposon           | Q488             |
| E8     | transposon           | I487             |
| F11    | transposon           | G485             |
| C5     | transposon           | N489             |

Table 1c: Mutants with intermediate IL-8-releasing activity

| Sample | Type of mutant | Site of mutation | Approx. EC$_{50}$ (µg/ml) |
|--------|----------------|------------------|---------------------------|
| del o  | deletion       | I13-L61          | 4                         |
| D10    | transposon     | S435             | 4                         |
| E9     | transposon     | T296             | 0.6                       |
| 2D12   | transposon     | V537             | 10                        |
| 2C3    | transposon     | E79              | 1.4                       |
Table 2: Activities of flagellins altered in the N-terminal conserved peptide region

| Sample      | Sequence                                      | Plant response | IL-8 response |
|-------------|-----------------------------------------------|----------------|---------------|
| Flg22       | QRLSTGSRINSAKDDAAGLQIA                       | ++             | 0             |
| Flg15<sub>EC</sub> | RINSAKDDAAGQAIA                                | +              | 0             |
| Flg15<sub>R. MEL.</sub> | RVGQAADNAAYWSIA                                | 0              | ND            |
| FliC-EAEC   | ...ERLSSGLRINSAKDDAAGQAIA...                 | ND             | ++            |
| FliC-S.t.   | ...ERLSSGLRINSAKDDAAGQAIA...                 | ND             | ++            |
| ΔR mel.     | ...ERLSSGLRVGQAKDDAAGQAIA...                 | ND             | ++            |
| ΔQA         | ...ERLSSGLRINSAKDDAAGLQIA...                 | ND             | ++            |
| G47A        | ...ERLSSGLRINSAKDDAALQIA...                  | 0′             | ++            |

Plant response: as reported in (14) and (16). IL-8 response: ability to release IL-8 from Caco-2 cells as described in Methods. ++ = full response; + reduced potency compared to wild-type; 0 = no activity; ND = not tested; 0′ = 22-mer with sequence given was inactive in the plant model; full-length flagellin containing this peptide not tested in this system.
Figure 1

MAQVINTNSLSLTQNNINQNQSLSSSIIERLLSSLRLINSAKDAAQQAIA
NRFTSNIKLTQARANNDGVISVAQTTEGALSEINNNLQRIRELTVQATTG
TNSDSDLSDIQDEIKSLDLIDIRVSGQTQFNGVNLSDKGSMIQVGAING
ETITDLKIHDSDLNLAGFNVEGETANTAALKDMVGLKGLDNTGVTAA
GVNRHIAKAVASSDILNAVGVDGSKVSTEADVFGAAAPGTPEYTYYH
KDTNTYTAASVDAFLQANPAGTTAAATVSIGGTTAQEQKVIIAKD
GSLTADDGAALYLDGTNKLTAAGTDQAKLSDLMNANAKTVITTDK
GFTANTTLDVCGDVISDASFTPANAVKNETYTAVGVTLPATYVNNGTA
SAYLVGKSTPAEFYHADGTITSGENATSKAIYVSANGNLTTNTTSE
SEATTNPLAALDDAIASIDKFSSLGAIQNRDLSAVTNLNTTNTNLSEAQS
RIQDADYATEVSNMSKAIQIIQQAGNSVLAKANQVFQVLSLQQG
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