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Analysis of *Escherichia coli* Colonization Factor Antigen I Linear B-Cell Epitopes, as Determined by Primate Responses, following Protein Sequence Verification

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Colonization factor antigen I (CFA/I)-bearing strains of enterotoxigenic *Escherichia coli* (ETEC) are responsible for a significant percentage of ETEC diarrheal disease worldwide whether the disease presents as infant diarrhea with high mortality or as traveler's diarrhea. CFA/I pili (fimbriae) are virulence determinants that consist of repeating protein subunits (pilin), are found in several ETEC serogroups, and promote attachment to human intestinal mucosa. While CFA/I pili are highly immunogenic, the antigenic determinants of CFA/I have not been defined. We wished to identify the linear B-cell epitopes within the CFA/I molecule as determined by primate response to the immunizing protein. To do this, we (i) resolved the discrepancies in the literature on the complete amino acid sequence of CFA/I by N-terminal and internal protein sequencing of purified and selected proteolytic fragments of CFA/I, (ii) utilized this sequence to synthesize 140 overlapping octapeptides covalently attached to polyethylene pins which represented the entire CFA/I protein, (iii) immunized three rhesus monkeys with multiple intramuscular injections of purified CFA/I subunit in Freund's adjuvant, and (iv) tested serum from each monkey for its ability to recognize the octapeptides in a capture enzyme-linked immunosorbent assay. Eight linear B-cell epitopes were identified; the region containing an epitope at amino acids 11 to 21 was strongly recognized by all three individual rhesus monkeys, while the amino acid stretches 22 to 29, 66 to 74, 93 to 101, and 124 to 136 each contained an epitope that was recognized by two of the three rhesus monkeys. The three other regions containing epitopes were recognized by one of the three individuals. The monkey antiserum to pilus subunits recognized native intact pili by immunogold labeling of CFA/I pili present on whole H10407 cells. Therefore, immunization with pilus subunits induces antibody that clearly recognizes both synthetic linear epitopes and intact pili. We are currently studying the importance of these defined epitope-containing regions as vaccine candidates.

Enterotoxigenic *Escherichia coli* (ETEC) strains are causative agents of diarrheal disease throughout the world, with symptoms ranging from a mild self-limiting diarrhea to a choleralike life-threatening disease (8, 35). Infant mortality due to ETEC in developing countries is estimated at almost 800,000 per year (2), while adult travelers to these regions are at risk to contract the illness (30). Colonization factor antigens (CFA) of ETEC have been demonstrated to be important in the initial step of colonization, adherence of the bacterium to intestinal epithelia (26). The first CFA to be described, CFA/I (11), is routinely found in large percentages of ETEC in epidemiological studies of adults and children with diarrhea (9, 27). CFA/I is present on the surfaces of bacteria in ETEC serogroups O4, O15, O25, O63, O78, O90, O110, O114, O126, O128, and O153 (40) in the form of pili (fimbriae), which are rigid, 7-nm diameter protein fibers composed of repeating pilin subunits. CFA/I promotes mannose-resistant attachment to human brush borders (6) with an apparent sialic acid sensitivity (12, 33); a vaccine that establishes immunity against this protein may prevent the attachment to host tissues and subsequent disease. Experimental immunization with CFA/I has protected against challenge with virulent CFA/I-bearing strains (10). In addition, because the CFA/I structural subunit shares N-terminal amino acid sequence homology with the coli surface-associated antigens CS1, CS2, and CS4 (16, 23, 44), a vaccine which contained cross-reactive epitopes from this area of the molecule might protect against infection with non-CFA/I-bearing ETEC.

Antigenic sites (B-cell epitopes) of protein antigens can be either continuous (made up of conformationally independent continuous surface portions of the polypeptide chain, i.e., sequential or linear epitopes) or discontinuous (spatially adjacent surface residues that are not in direct peptide bond linkage either partially or totally) (1). Until recently, experiments to identify linear B-cell epitopes were time-consuming, tedious, and costly; however, technology which allows simultaneous identification of all linear B-cell epitopes in the protein of interest is now available. Geysen et al. (14) developed a technique for the simultaneous synthesis of hundreds of peptides on polyethylene pins (Pepscan). Peptides covalently attached to the pins can be used directly in enzyme-linked immunosorbent assays (ELISA), and the technique has been successfully used to define linear B-cell epitopes of proteins from viruses (14, 29), bacteria (19), parasites (32), and eucaryotes (41, 42). Thus, this technology allows efficient mapping and localization of linear B-cell epitopes from essentially any protein of known sequence and can be utilized to resolve epitopes to a single amino acid (14).

A critical problem to be dealt with before synthesis of CFA/I peptides could begin was discrepancies between the three published amino acid sequences of CFA/I. The primary structure of CFA/I has been previously determined by

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protein-sequencing techniques (22) and by molecular cloning methods (17, 21), with agreement in all but 3 of the 147 amino acid residues in these three studies.

This study was designed to reexamine the primary structure of CFA/I by resolving the dispute about CFA/I amino acids in the literature; to utilize this sequence for synthesis of all 140 possible overlapping CFA/I-derived octapeptides, and to use these covalently attached peptides to identify linear B-cell epitopes of CFA/I with sera from rhesus monkeys immunized with CFA/I subunits. In addition, we demonstrated that the serum that recognizes linear epitopes will also recognize native intact CFA/I.

MATERIALS AND METHODS

CFA/I purification. Intact CFA/I pili were purified from H10407 (O78:H^-) as described by Hall et al. (16). Briefly, bacteria grown on CFA agar were subjected to shearing, with the shearate subjected to differential centrifugation and isopycnic banding on cesium chloride in the presence of *N*-laurylsarcosine. CFA/I pili were dissociated to free subunits in 6 M guanidinium HCl–0.2 M ammonium bicarbonate (2 h, 25°C) and passed through an ultrafiltration membrane (XM 50 stirred cell; Amicon, Danvers, Mass.), with concentration and buffer exchange to phosphate-buffered saline (PBS), pH 7.2, on a YM 10 stirred cell (Amicon). Examination of dissociated pili by electron microscopy demonstrated a lack of pilus structure, whereas intact pili were evident prior to dissociation (not shown).

Protein sequencing. The N-terminal sequence of purified intact CFA/I was obtained by pipetting 0.4 to 0.7 nmol of CFA/I in PBS onto a precycled Polybrene membrane and subjecting the protein to gas-phase sequencing (Applied Biosystems 470, Foster City, Calif.). In order to determine the internal protein sequence, the covalent structure of CFA/I was disrupted by enzymatic digestion. Trypsin or *Staphylococcus aureus* V8 protease (V8) (sequencing grade; Boehringer Mannheim) was incubated with intact CFA/I at a 1:50 (wt/wt) ratio (Tris 50 mM, 0.1% sodium dodecyl sulfate [SDS], pH 8.5, for 16 h at 37°C [trypsin] or 24°C [V8]). Digested material was loaded onto precast 16% Tricine SDS-polyacrylamide gels (37) (NOVEX, Encinitas, Calif.) and run according to the manufacturer's instructions. Separated samples were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Westrans; Schleicher & Schuell, Keene, N.H.) by the method of Matsudaira (31) and with the NOVEX miniblot apparatus. Blotted proteins were stained with rapid Coomassie stain (Diversified Biotech, Newton Center, Mass.). To obtain the desired fragment containing the residue of interest within a region accessible by automated gas-phase sequencing techniques (preferably 15 or fewer residues), molecular weights of fragments were estimated from SDS-polyacrylamide gel electrophoresis (PAGE) gels by comparing fragment mobilities with standard mobilities in the same gel (standard molecular weights of 20,400 to 2,512; trypsin inhibitor, myoglobin, and myoglobin cyanogen bromide [CNBr] fragments; Diversified Biotech). The corrected molecular weights for the myoglobin CNBr fragments as reported in Kratzin et al. (25) were used. The estimated molecular weights for the unknown CFA/I fragments from SDS-PAGE gels were compared with calculated molecular weights of CFA/I fragments predicted from the same enzyme treatment (using PeptideSort, developed by the University of Wisconsin Genetics Computer Group [7]). Fragments selected in this manner were excised from the PVDF membrane and subjected to gas-phase sequencing.

Monkey immunization. Three rhesus monkeys (*Macaca mulatta*) weighing 7.3 to 7.5 kg each were injected intramuscularly with 250 μg of dissociated CFA/I in complete Freund's adjuvant and subsequently with two injections of 250 μg each of the same antigen in incomplete Freund's adjuvant at weekly intervals. Blood was drawn prior to and 3 weeks after primary immunization.

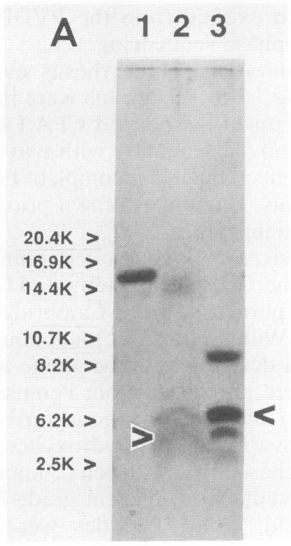
Peptide synthesis. Continuous overlapping peptides were synthesized by the Geysen pin method (14), with blocks of derivatized pins purchased from Cambridge Research Biochemicals, Inc. (Wilmington, Del.) and equipment and software of our own design (4). Fmoc-amino acid pentafluorophenyl esters were purchased from Peninsula Laboratories (Belmont, Calif.) and used without further treatment or analysis. The activating agent 1-hydroxybenzotriazole monohydrate was purchased from Aldrich Chemical Co. (Milwaukee, Wisc.). Solvents were reagent grade from Fisher Scientific (Springfield, N.J.). Peptides were synthesized as octamers and remained linked to the resin. Peptide composition was confirmed by amino acid analysis of one control pin per plate. Pins were hydrolyzed in 6 N HCl at 110°C for 24 h, and the analysis was performed on a Beckman 6300 amino acid analyzer.

ELISA procedure. The capture ELISA of Geysen et al. (14) was utilized, with minor modifications. After an initial blocking step (60 min, room temperature, in PBS–0.1% Tween 20–1% casein–1% bovine serum albumin), sera raised in rhesus monkeys to purified dissociated pili were incubated with one set of pins with peptides representing the entire CFA/I. The serum was diluted at 1:1,000, and the preimmune serum of the same animal, which was tested at an identical dilution, was simultaneously incubated with a duplicate set of pins. The pins were incubated with primary antibody at 4°C for 16 h and washed three times, and secondary antibody (goat anti-human immunoglobulin G [heavy and light chains], alkaline phosphatase labeled [Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.]), was added at a 1:500 dilution (60 min, room temperature) and washed three times. Substrate solution was added (10% diethanolamine, 0.05% *p*-nitrophenylphosphate [Sigma], pH 9.8), and the A_{405} was measured on a Dynatech MR 5000 or MR 580. Before reuse of the pins, bound protein was removed by bath sonication (30 min, 70°C in PBS–1% SDS–0.2% 2-mercaptoethanol), hot water rinse, and boiling-methanol treatment (65°C, 2 min).

Immunoelectron microscopy (IEM). Bacterial suspensions were spread over the surface of Formvar-carbon-coated grids. Each grid was placed on a drop of primary antiserum diluted in Dulbecco's PBS (Advanced Biotechnologies, Inc., Columbia, Md.) with 1% bovine serum albumin (Sigma) (DPBS-B), incubated for 20 min at room temperature, and washed by sequentially placing the grid on six drops of DPBS-B. Then each grid was placed on a drop of 10-nm-diameter protein G-gold (Structure Probe Inc., West Chester, Pa.) diluted in DPBS-B for 20 min. Final washing was carried out on 4 drops of DPBS and 2 drops of ultrapure water. Samples were then negatively stained with 2% ammonium molybdate (Sigma) and examined in a JEOL model 100B electron microscope at 80 kV.

RESULTS

Verification of the amino acid sequence of the CFA/I pilin subunit. It was essential to utilize the appropriate amino acid



B

Lane 2 LADTPQLTDVLNSTVQMP (62-79)

Lane 3 SYRVMTQVHTNDATKKVIV (42-60)

FIG. 1. (A) SDS-PAGE of intact CFA/I (lane 1), trypsin-treated CFA/I (lane 2), and *S. aureus* V8-treated CFA/I (lane 3). Molecular masses of individual bands were estimated from molecular weight standards (on left). Additional gels with three lanes each of both the trypsin- and V8-treated CFA/I (approximately 10 μg of protein per lane) were transferred to PVDF membranes, where the bands corresponding to the approximate molecular masses of 3,500 (tryptic digest; arrow, lane 2) and 6,000 (V8 digest; arrow, lane 3) were excised and subjected to Edman degradation. (B) Resulting sequence of protein fragments from each lane of A (residue numbers of the sequenced portion of fragment in the intact protein). Underlined, italicized residues are amino acids under dispute in the literature.

sequence of CFA/I in the peptide synthesis to carry out the studies as planned. At issue were the amino acids at position 14, 53, and 74; incorrect residues at those positions would effect 24 of 140 pins (17%). To resolve the discrepancy in the literature, intact purified as well as selected fragments of proteolytically digested CFA/I were subjected to N-terminal sequencing. N-terminal sequencing of the intact protein was performed in order to determine the identity of residue 14. Twenty-nine residues of the protein were determined (not shown), with residue 14 present as valine. All other amino acids sequenced were in agreement with the three published reports. The enzymes trypsin and V8 were chosen in order to give proteolytic fragments with the residues of interest (53 and 74) relatively near the fragment N terminus for automated Edman degradation of the fragment from the PVDF membrane. These digests were separated on Tricine SDS-PAGE gels (Fig. 1A), and the molecular masses of the fragments were estimated. Computer analysis of CFA/I determined that a fragment of 3,459 Da, corresponding to amino acids 62 to 94, was expected from the tryptic digest, and a fragment of 5,889 Da, residues 42 to 95, was expected from the V8 digest. The putative fragments were located within each digest (arrows in Fig. 1A), additional gels with multiple lanes of each digest were run, the fragments were transferred to a PVDF membrane, and the selected bands were excised and sequenced. N-terminal sequences of each fragment are given in Fig. 1B. The N-terminal 18 residues from the tryptic fragment (positions 62 to 79) were determined. Thirteen residues into the fragment (analogous to position 74 of the intact protein), the amino acid was serine. Nineteen residues of the V8 fragment, corresponding to residues 42 to 60 of the parent protein, were determined. Residue 12 of the fragment (amino acid 53 of CFA/I) was present as aspartic acid. By including all three sequences, we determined the primary structure of 66 amino acids (41% of CFA/I); all areas sequenced encompass residues disputed in the literature. For the following peptide synthesis, we therefore utilized the complete amino acid sequence of CFA/I as given by Karjalainen et al. (21). The calculated molecular weight for CFA/I with this revised sequence is 15,057.

Determination of B-cell epitopes within the CFA/I pilin subunit. Sera from rhesus monkeys immunized with CFA/I subunits were tested in the capture ELISA (Fig. 2), with the preimmunization sera tested simultaneously at an identical dilution on duplicate pins. The peptides considered positive were defined as those peaks which clearly stood out above the background and had little or no increase in absorbance compared with the preimmune serum of the same animal. Monkey 2Z2 (Fig. 2A and D) responded strongly to six regions of the CFA/I sequence. Peptide 14 (the octapeptide encompassing amino acids 14 to 21) gave the strongest response, and the peptides on the four pins adjacent to it (numbers 11, 12, 13, and 15) also appeared to bind significant antibody. The other 2Z2-defined regions containing epitopes are found at peptides 3 to 4, 22, 32 to 33, 93, and 124. Monkey 184D (Fig. 2B and E) also responded strongly to peptide 14, although the maximum response was to peptide 13, with strong involvement of peptide 12 in the epitope region. Additional peptides recognized by monkey 184D were seen at peptide numbers 22, 38, 66, and 93 to 94. Monkey 34 serum also reacted to the region of the CFA/I primary structure near the N terminus, but the response occurred at peptides 11, 12, and, weakly, 14. The two other regions containing epitopes identified by monkey 34 were found within the peptides 65 to 67 and 127 to 129. The revised primary structure of CFA/I with recognized regions as defined by the response of the three individual rhesus monkeys is illustrated in Fig. 3. Figures 2 and 3 show that different individuals subjected to identical immunization protocols have varied responses to CFA/I. The region encompassing amino acids 11 to 21 contained the only epitope to be recognized by all three monkeys. The epitopes within residues 93 to 101 and 22 to 29 were recognized by monkeys 2Z2 and 184D, while residues 124 to 136 were identified by monkeys 2Z2 and 34, and the epitope within amino acids 66 to 74 was recognized only by monkeys 184D and 34. Each of the remaining three regions containing epitopes (32 to 40, 38 to 45, and 3 to 11) was recognized by a single individual only (2Z2, 184D and 2Z2, respectively). Three amino acids (KTF) overlap between the 32-to-40 and 38-to-45 regions recognized by 2Z2 and 184D, respectively. Results from the duplicate pins incubated with the preimmune sera (Fig. 2D, E, and F) show a relatively low background with little or no preexisting specific antibody to the peptides. Background response to regions not involved in epitopes increased considerably after immunization in rhesus monkeys 2Z2 and 184D only.

IEM. Hyperimmune serum from each of the three monkeys was utilized as the source of primary antibody in IEM of CFA/I-bearing H10407 (Fig. 4A to C). Sera from each of

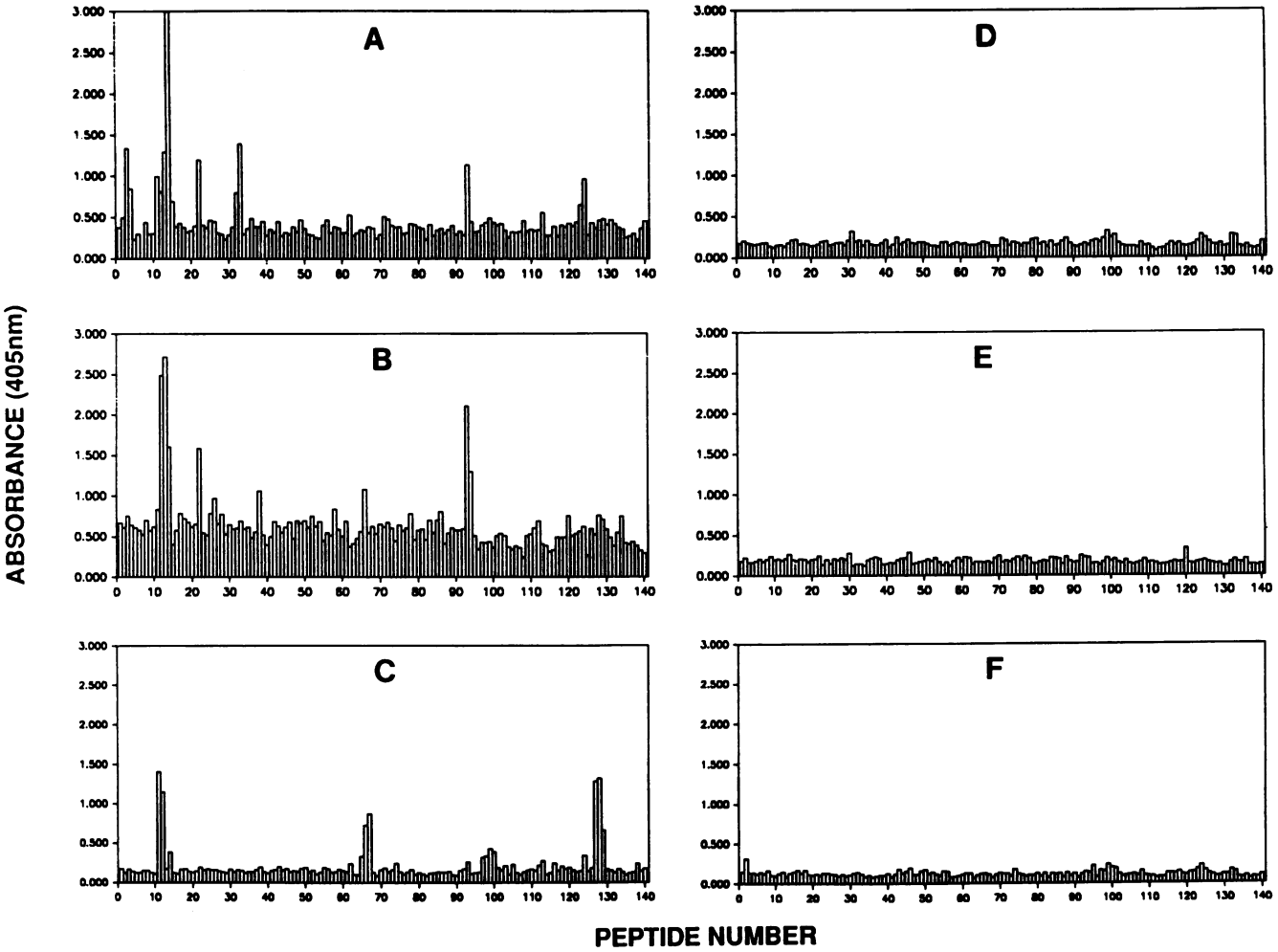


FIG. 2. ELISA results of testing sera of rhesus monkeys 2Z2 (A, D), 184D (B, E), and 34 (C, F) hyperimmune (A, B, C) and preimmune (D, E, F) to CFA/I subunits against a complete set of octapeptides synthesized on polyethylene pins that represent the entire CFA/I primary structure. Monkey sera were diluted 1:1,000. Peptide number refers to the first amino acid in the sequence of each octapeptide on each pin from the CFA/I primary structure.

the three monkeys gave similar results, with all showing a labeling of gold particles along the pilus shaft at several places with no apparent periodicity. None of the sera appeared to cause a cross-linking or agglutination of pili at higher or lower serum concentrations. Control nonimmune serum (Fig. 4D) under identical conditions demonstrated a lack of reactivity to the H10407 bacterial cell in general and to the CFA/I pili in particular.

DISCUSSION

With a strategy designed to determine the individual residues in question, N-terminal and internal protein sequencing of CFA/I in this report yielded the sequence of 66 of the 147 total residues (41%) and determined that for the CFA/I protein studied, residues 14, 53, and 74 were valine, aspartic acid, and serine, respectively. The amino acid sequence of CFA/I has been presented in three published reports (17, 21, 22), with agreement on the majority of the overall sequence and the total number of amino acids. The disputed residues were 14, 53, and 74. By using protein sequencing techniques, Klemm (22) found these residues to be valine, asparagine, and alanine, respectively. Two groups have sequenced the DNA encoding CFA/I. The deduced amino acid sequence from Hamers et al. (17) gave alanine, aspartic acid, and serine, respectively, while Karjalainen et al. (21) reported valine, aspartic acid, and serine, respectively. The single amino acid difference between Karjalainen et al. (21) and Hamers et al. (17) at position 14 is the result of a difference at a single base (GCA versus GTA, respectively). In addition, Hall et al. (16) presented the N-terminal 20 amino acid residues of CFA/I with a valine reported at residue 14. The differences reported in these studies may be attributable to strain variation, mutation, or sequencing errors. The actual reasons for these differences notwithstanding, it was of great importance to the present study to utilize the appropriate amino acid sequence in the peptide synthesis, specifically that of the actual protein to which we were raising antisera. This strategy of selective proteolytic digestion, separation of fragments by SDS-PAGE, and microsequence analysis of specific predicted fragments may prove useful in other cases when disagreements in published sequence of the same protein occurs. There is an additional

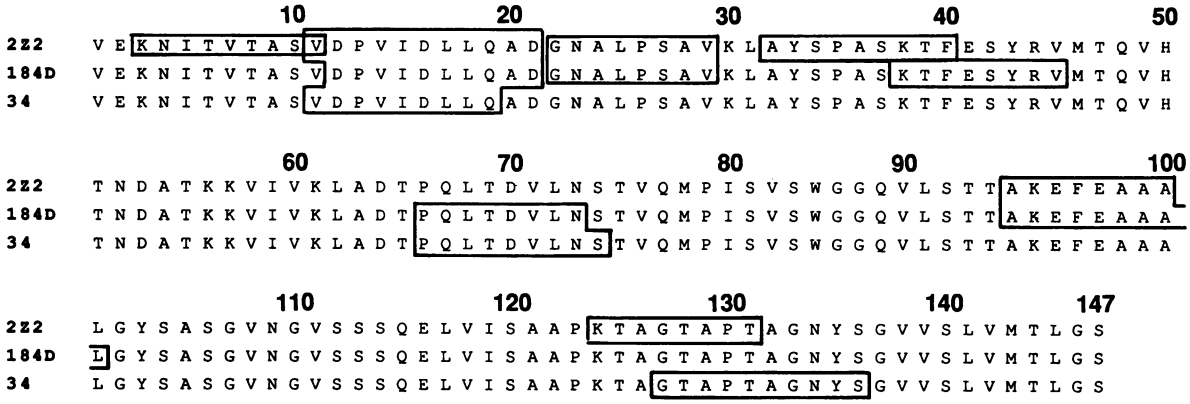


FIG. 3. Complete revised sequence of CFA/I (147 amino acids) with antigenic determinants (boxed areas) as defined by each individual monkey response (2Z2, 184D, and 34). Figure derived from data in Fig. 2.

example of a discrepancy within the sequences of ETEC pili for which this method might be useful. Amino acid 61 of the major subunit of CS3 pili has been reported as serine (bases AGT) (3) and asparagine (AAT) (18). All other amino acids in these reports are in agreement.

Eight linear B-cell epitopes were identified by the immune response of three rhesus monkeys to dissociated CFA/I pili. All three monkeys recognized the region containing an epitope encompassing residues 11 to 21. Four epitope-containing regions were recognized by two of the three individuals, and three were recognized by a single monkey. Individual differences within a species as well as differences between species in antibody responses to linear peptides is commonly reported (15, 34). With respect to differences between species, epitopes defined in rhesus monkeys will most likely be of greater value for human studies than epitopes defined in lower vertebrate species. With an ultimate goal of development of peptide-based ETEC vaccines for humans, monkey-defined epitopes should give the greater chance of successful epitope selection. At this point, it is not possible to define the exact boundaries and critical amino acids of each epitope within the regions identified. Analysis of peptides wherein each amino acid of the region is systematically replaced allows this definition and could result in a modified peptide that binds significantly more antibody than do peptides derived from the native protein (15).

In this study, pili dissociated into subunits were used to immunize rhesus monkeys. In our experience with *E. coli* AF/R1 pili (5), immunization with intact AF/R1 produced little or no response to linear epitopes, whereas sera derived from immunization with AF/R1 subunits gave a strong response to linear epitopes. No response to linear epitopes by Pepscan analysis was found on immunization with intact *E. coli* K88, despite a strong response to the intact pili (43). From these studies, it appears that immunization with dissociated pili is necessary to get a response to linear peptides and that conformational epitopes are immunodominant in the three *E. coli* pili studied to date.

While immunization with CFA/I subunits resulted in antibody to linear epitopes, it also yielded antibody binding to intact pili as demonstrated by IEM. This same phenomenon has also been shown with AF/R1 pili (5). These results are in contrast to those of Karch et al. (20), who found by using IEM that antisubunit antibodies did not adhere to intact *E. coli* WF96 pili. With CFA/I and AF/R1, the population of antibodies binding to the intact pili may not necessarily be the same population of antibodies binding to the linear epitopes, but it has been demonstrated that peptide-specific antibodies can bind to native pili, such as *E. coli* P pili (39), or to native multisubunit toxins, as has been demonstrated with the S3 subunit of pertussis toxin (38). Additional studies are necessary to identify the specific epitopes to which antibody is binding, whether linear or conformational.

Five of the eight CFA/I epitopes fall within the first 45 amino acids, and six epitopes fall within the N-terminal 50% of CFA/I. Lopez-Vidal et al. (28) developed monoclonal antibodies (MAb) to CFA/I and examined whether a series of soluble proteolytic fragments representing the entire CFA/I protein would inhibit binding of two of the MAb to solid-phase CFA/I. The only fragment to effectively inhibit both MAb encompassed residues 1 to 46. These data are consistent with our finding that the majority of the linear epitopes are present in the first 45 residues, although the fine specificity of the MAb has not been reported, i.e., whether the specificity is to a specific continuous or a discontinuous epitope(s) within the fragment. Of additional interest in the study by Lopez-Vidal et al. (28) was the finding that one of the two MAb inhibited the binding of CFA/I-bearing bacteria to blood group A erythrocytes, illustrating the concept of adherence-blocking antipilus antibody.

Klemm and Mikkelsen (24) predicted potential antigenic determinants of CFA/I based on computer analysis of the amino acid sequence of Klemm (22). Six sites were chosen and ranked on the basis of their predicted existence in a hydrophilic stretch and having a reverse turn at or near this stretch. By these criteria, six sites were chosen in their order of potential as a linear epitope: 52 to 57, 61 to 66, 37 to 42, 112 to 116, 19 to 24, and 93 to 99. The best and only true match came with the least likely predicted epitope (93 to 99), which matched the experimentally determined epitope within the region 93 to 101 very well. The epitopes predicted at 19 to 24 and 37 to 42 each overlap with two experimentally determined epitope regions (11 to 21 with 22 to 29, and 32 to 40 with 38 to 42, respectively). The remaining three epitopes had no overlap with any of the experimentally determined epitopes presented herein except that epitope 61 to 66 (predicted) overlapped by only 1 amino acid the epitope contained in amino acids 66 to 74 (experimental). The predicted epitope at amino acids 52 to 57 contained residue 53, reported elsewhere (22) as asparagine. Asparagine is considered to contribute significantly to reverse turns and

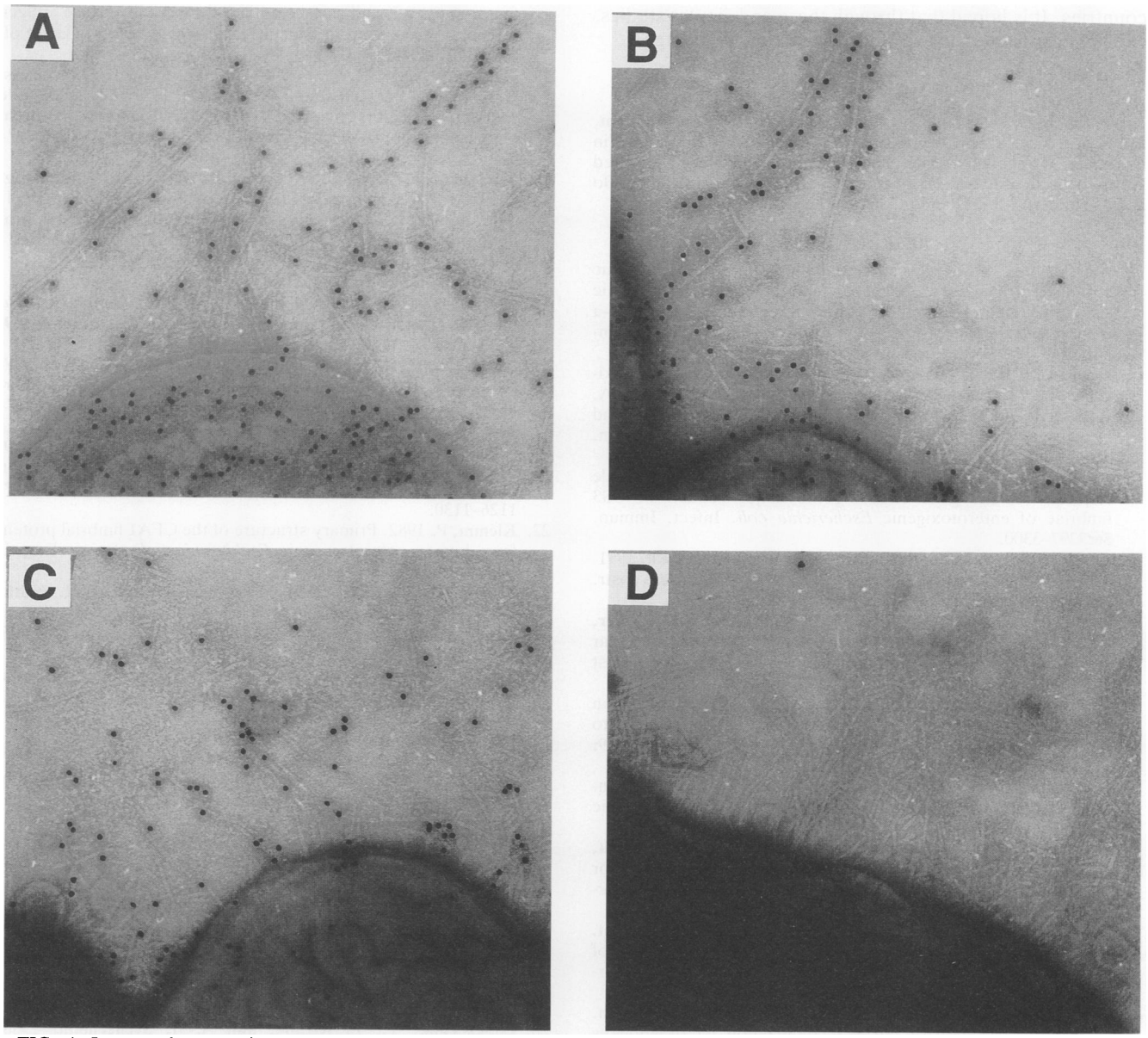


FIG. 4. Immunoelectron microscopy of the three hyperimmune monkey sera reacted with CFA/I-bearing H10407 (O78:H$^-$). Monkeys were 2Z2 (A), 184D (B), and 34 (C). All sera were tested at a 1:20 dilution and then treated with protein G-gold (10 nm) diluted 1:20. (D) Control nonimmune serum of monkey 34 under identical conditions. See Materials and Methods for additional details. Magnification, ×113,000.

gives the prediction more validity. In this report, an aspartic acid was found at residue 53, which was used for peptide synthesis, and this region was found not to bind significant antibody. Predictive methods based on averaged hydrophilicity may not identify a number of epitopes, since hydrophobic and hydrophilic amino acids are found in linear B-cell epitopes (15). When evaluating three predictive methods, Getzoff et al. (13) found that the methods were not highly successful when evaluated against peptide mapping results or against the Geysen pin technology (36). While predictive methods can be useful, the ability to scan the entire length of a protein in the Pepscan method is a very powerful, relatively facile experimental determination of all of the actual linear B-cell epitopes of a protein.

We have shown that linear B-cell epitopes within CFA/I can be identified and localized by the primate immune response using immobilized CFA/I-derived synthetic peptides and that the same serum recognizes native intact CFA/I. By an analysis using decapeptides chemically removed from a similar set of polyethylene pins, we will report on the T-cell epitopes of CFA/I as defined by the response of the same rhesus monkeys in this report (18a). At present, we are examining proteolytic fragments of CFA/I in further epitope analysis, elucidating the minimal binding epitopes and essential amino acids of each CFA/I linear B-cell epitope, and synthesizing and purifying peptides containing the epitopes defined in this study for animal immunization experiments. ETEC continues to be responsible for substantial infant mortality in developing countries and is the major pathogen responsible for diarrhea in travelers visiting these

countries. It is hoped that through these and similar studies, effective candidates for an ETEC vaccine will emerge.

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REFERENCES

1. **Atassi, M. Z.** 1978. Precise determination of the entire antigenic structure of lysozyme: molecular features of protein antigenic structures and potential of 'surface-simulation' synthesis—a powerful new concept for protein binding sites. Immunochemistry **15:**909–939.
2. **Black, R. E.** 1986. The epidemiology of cholera and enterotoxigenic *E. coli* diarrhea disease, p. 22–32. *In* J. Holmgren, A. Lindberg, and R. Möllby (ed.), Development of vaccines and drugs against diarrhea. 11th Nobel Conference, Stockholm. Studentlitteratur, Lund, Sweden.
3. **Boylan, M., C. J. Smyth, and J. R. Scott.** 1988. Nucleotide sequence of the gene encoding the major subunit of CS3 fimbriae of enterotoxigenic *Escherichia coli*. Infect. Immun. **56:**3297–3300.
4. **Carter, J. M., J. Lyon, S. VanAlbert, J. Lee, and C. Deal.** 1991. The platemate: an aid to Geysen peptide pin syntheses, abstr. 275. Program Abstr. 12th Am. Peptide Sympos.
5. **Cassels, F. J., B. H. Byun, J. L. Nauss, R. H. Reid, J. M. Carter, C. D. Deal, and E. C. Boedeker.** 1991. Identification of linear B-cell epitopes of *E. coli* pili, abstr. B-141, p. 49. Abstr. 91st Gen. Meet. Am. Soc. Microbiol. 1991.
6. **Cheney, C. P., and E. C. Boedeker.** 1983. Adherence of an enterotoxigenic *Escherichia coli* strain, serotype O78:H11, to purified human intestinal brush borders. Infect. Immun. **39:** 1280–1284.
7. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
8. **Echeverria, P., J. Seriwatana, D. N. Taylor, S. Changchawalit, C. S. Smyth, S. Towbig, and B. Rowe.** 1986. Plasmids coding for colonization factor antigens I and II, LT and ST-A2 in *Escherichia coli*. Infect. Immun. **51:**626–630.
9. **Evans, D. G., D. J. Evans, Jr., W. S. Tjoa, and H. L. DuPont.** 1978. Detection and characterization of colonization factor of enterotoxigenic *Escherichia coli* isolated from adults with diarrhea. Infect. Immun. **19:**727–736.
10. **Evans, D. G., D. Y. Graham, D. J. Evans, Jr., and A. Opekun.** 1984. Administration of purified colonization factor antigens (CFA/I, CFA/II) of enterotoxigenic *Escherichia coli* to volunteers. Response to challenge with virulent enterotoxigenic *Escherichia coli*. Gastroenterology **87:**934–940.
11. **Evans, D. G., R. P. Silver, D. J. Evans, Jr., D. G. Chase, and S. L. Gorbach.** 1975. Plasmid controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. **12:**656–661.
12. **Faris, A., M. Lindahl, and T. Wadström.** 1980. GM_2-like glycogconjugate as possible erythrocyte receptor for the CFA/I and K99 haemagglutinins of enterotoxigenic *Escherichia coli*. FEMS Microbiol. Lett. **7:**254–269.
13. **Getzoff, E. D., J. A. Tainer, R. A. Lerner, and H. M. Geysen.** 1988. The chemistry and mechanism of antibody binding to protein antigens. Adv. Immunol. **43:**1–98.
14. **Geysen, H. M., R. H. Meloen, and S. J. Barteling.** 1984. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc. Natl. Acad. Sci. USA **81:**3998–4002.
15. **Geysen, H. M., J. A. Tainer, S. J. Rodda, T. J. Mason, H. Alexander, E. D. Getzoff, and R. A. Lerner.** 1987. Chemistry of antibody binding to a protein. Science **235:**1184–1190.
16. **Hall, R. H., D. R. Maneval, J. H. Collins, J. L. Theibert, and M. M. Levine.** 1989. Purification and analysis of colonization factor antigen I, coli surface antigen 1, and coli surface antigen 3 fimbriae from enterotoxigenic *Escherichia coli*. J. Bacteriol. **171:**6372–6374.
17. **Hamers, A. M., H. J. Pel, G. A. Willshaw, J. G. Kusters, B. A. M. van der Zeijst, and W. Gaastra.** 1989. The nucleotide sequence of the first two genes of the CFA/I fimbrial operon of human enterotoxigenic *Escherichia coli*. Microb. Pathog. **6:**297–309.
18. **Jalajakumari, M. B., C. J. Thomas, R. Halter, and P. Manning.** 1989. Genes for biosynthesis and assembly of CS3 pili of CFA/II enterotoxigenic *Escherichia coli*: novel regulation of pilus production by bypassing an amber codon. Mol. Microbiol. **3:**1685–1695.

18a. **Jarboe, D. L., et al.** Unpublished data.

19. **Joys, T. M., and F. Schödel.** 1991. Epitope mapping of the *d* flagellar antigen of *Salmonella muenchen*. Infect. Immun. **59:** 3330–3332.
20. **Karch, H., H. Leying, P. Goroncy-Bermes, H.-P. Kroll, and W. Opferkuch.** 1985. Three-dimensional structure of fimbriae determines specificity of immune response. Infect. Immun. **50:**517–522.
21. **Karjalainen, T. K., D. G. Evans, M. So, and C. H. Lee.** 1989. Molecular cloning and nucleotide sequence of the colonization factor antigen I gene of *Escherichia coli*. Infect. Immun. **57:** 1126–1130.
22. **Klemm, P.** 1982. Primary structure of the CFA1 fimbrial protein from human enterotoxigenic *Escherichia coli* strains. Eur. J. Biochem. **124:**339–348.
23. **Klemm, P., W. Gaastra, M. M. McConnell, and H. R. Smith.** 1985. The CS2 fimbrial antigen from *Escherichia coli*: purification, characterization and partial covalent structure. FEMS Microbiol. Lett. **26:**207–210.
24. **Klemm, P., and L. Mikkelsen.** 1982. Prediction of antigenic determinants and secondary structures of the K88 and CFA1 fimbrial proteins from enteropathogenic *Escherichia coli*. Infect. Immun. **38:**41–45.
25. **Kratzin, H. D., J. Wiltfang, M. Karas, V. Neuhoff, and N. Hilschmann.** 1989. Gas-phase sequencing after electroblotting on polyvinylidene difluoride membranes assigns correct molecular weights to myoglobin molecular weight markers. Anal. Biochem. **183:**1–8.
26. **Levine, M. M., J. B. Kaper, R. E. Black, M. L. Clements.** 1983. New knowledge on pathogens of bacterial enteric infections as applied to vaccine development. Microbiol. Rev. **47:**510–550.
27. **Lopez-Vidal, Y., J. J. Calva, A. Trujillo, A. Ponce de León, A. Ramos, A.-M. Svenerholm, and G. M. Ruiz-Palacios.** 1990. Enterotoxins and adhesins of enterotoxigenic *Escherichia coli*: are they risk factors for acute diarrhea in the community? J. Infect. Dis. **162:**442–447.
28. **Lopez-Vidal, Y., P. Klemm, and A.-M. Svenerholm.** 1988. Monoclonal antibodies against different epitopes on colonization factor antigen I of enterotoxin-producing *Escherichia coli*. J. Clin. Microbiol. **26:**1967–1972.
29. **Lozzi, L., M. Rustici, M. Corti, M. G. Cusi, P. E. Valensin, L. Bracci, A. Santucci, P. Soldani, A. Spreafico, and P. Neri.** 1990. Structure of rubella E1 glycoprotein epitopes established by multiple peptide synthesis. Arch. Virol. **110:**271–276.
30. **MacDonald, K. L., and M. L. Cohen.** 1986. Epidemiology of traveler's diarrhea: current perspectives. Rev. Infect. Dis. **8:**S117–S121.
31. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidine difluoride membranes. J. Biol. Chem. **262:**10035–10038.
32. **Miles, M. A., G. R. Wallace, and J. L. Clarke.** 1989. Multiple peptide synthesis (Pepscan method) for the systematic analysis of B- and T-cell epitopes: application to parasite proteins. Parasitol. Today **5:**397–400.
33. **Pieroni, P., E. A. Worobec, W. Paranchych, and G. D. Armstrong.** 1988. Identification of a human receptor for colonization factor antigen I pili expressed by H10407 enterotoxigenic *Escherichia coli*. Infect. Immun. **56:**1334–1340.
34. **Rodda, S. J., H. M. Geysen, T. J. Mason, and P. G. Schoofs.** 1986. The antibody response to myoglobin. I. Systematic syn-

thesis of myoglobin peptides reveals location and substructure of species-dependent continuous antigenic determinants. Mol. Immunol. **23**:603–610.

35. **Sack, R. B.** 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. Annu. Rev. Microbiol. **26**:333–353.
36. **Saul, A. J., and H. M. Geysen.** 1990. Identification of epitopes through peptide technology, p. 117–126. *In* G. C. Woodrow and M. M. Levine (ed.), New generation vaccines. Marcel Dekker, Inc., New York.
37. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166**:368–379.
38. **Schmidt, M. A., P. O'Hanley, D. Lark, and G. K. Schoolnik.** 1988. Synthetic peptides corresponding to protective epitopes of digalactoside-binding pilin prevent infection in a murine pyelonephritis model. Proc. Natl. Acad. Sci. USA **85**:1247–1251.
39. **Schmidt, M. A., B. Raupach, M. Szulczynski, and J. Marzillier.** 1991. Identification of linear B-cell determinants of pertussis toxin associated with the receptor recognition site of the S3 subunit. Infect. Immun. **59**:1402–1408.
40. **Smith, H. R., S. M. Scotland, and B. Rowe.** 1985. Genetics of *Escherichia coli* virulence, p. 227–269. *In* M. Sussman (ed.), The virulence of *Escherichia coli*. Academic Press, Inc., New York.
41. **Tan, X. H., M. Ratnam, S. M. Huang, P. L. Smith, and J. H. Freisheim.** 1990. Mapping the antigenic epitopes of human dihydrofolate reductase by systematic synthesis of peptides on solid supports. J. Biol. Chem. **265**:8022–8026.
42. **Troalen, F., A. Razafindratsita, A. Puisieux, T. Voeltzel, C. Bohuon, D. Bellet, and J. M. Bidart.** 1990. Structural probing of human lutropin using antibodies raised against synthetic peptides constructed by classical and multiple antigen peptide system approaches. Mol. Immunol. **27**:363–368.
43. **van Zijderveld, F. G., J. Anakotta, R. A. M. Brouwers, A. M. van Zijderveld, D. Bakker, and F. K. de Graaf.** 1990. Epitope analysis of the F4 (K88) fimbrial antigen complex of enterotoxigenic *Escherichia coli* by using monoclonal antibodies. Infect. Immun. **58**:1870–1878.
44. **Wolf, M. K., G. P. Andrews, B. D. Tall, M. M. McConnell, M. M. Levine, and E. C. Boedeker.** 1989. Characterization of CS4 and CS6 antigenic components of PFC8775, a putative colonization factor complex from enterotoxigenic *Escherichia coli* E8775. Infect. Immun. **57**:164–173.