Characterization and Studies of the Cellular Interaction of Native Colonization Factor CS6 Purified from a Clinical Isolate of Enterotoxigenic Escherichia coli

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CS6 is a widely expressed colonization factor of enterotoxigenic Escherichia coli (ETEC). To date, CS6 has not been well characterized in its native state. Here, we purified CS6 for the first time from an ETEC clinical isolate. Purified CS6 was composed of two structural subunits, CssA and CssB, which were present in equal amounts and tightly linked through noncovalent, detergent-stable association. The CssA subunit was poorly immunogenic, whereas CssB was highly immunogenic. Although the predicted molecular mass of CssA is 15 kDa, the purified CssA has an effective molecular mass of 18.5 kDa due to fatty acid modification. When purified CS6 was screened for its ability to bind with different extracellular matrix proteins, fibronectin (Fn) was found to interact with CS6 as well as CssA in a dose-dependent and saturable manner. This interaction was inhibited both by a synthetic peptide corresponding to the C-terminal hydrophilic, surface-exposed region of CssA (positions 112 to 126) and by the antibody derived against this region. Enzyme-linked immunosorbent assay results showed that CssA interacted with the 70-kDa N-terminal domain of Fn. The modifications on CssA probably do not play a role in Fn binding. Preincubation of INT 407 cells with CssA, but not CssB, inhibited ETEC binding to these cells. The results suggested that CS6-expressing ETEC binds to Fn of INT 407 cells through the C-terminal region of CssA. Purified CS6 was found to colocalize with Fn along the junctions of INT 407 cells. Based on the results obtained, we propose that CS6-expressing ETEC binds to the intestinal cells through Fn for colonization.

Enterotoxigenic Escherichia coli (ETEC) infection is the leading cause of infantile diarrhea in developing countries and an important etiologic agent for traveler’s diarrhea. ETEC accounts for approximately 210 million diarrhea episodes and 380,000 deaths annually (35). Community-based studies conducted in developing countries with children younger than 5 years have shown that ETEC was the most frequently isolated enteropathogen (34, 35). As a cause of traveler’s diarrhea, ETEC was found to be associated with 40 to 70% of the cases, with drastic outcome in terms of morbidity and economic consequences (34).

In order to initiate pathogenesis, ETEC strains must adhere to the small intestine (14). This event is mediated by several proteinaceous surface antigens, collectively known as colonization factor antigens (CFAs) (6). To date, more than 25 distinct colonization factors have been identified, of which CS6 is the most prevalent in many countries (7, 20, 22). Many of the colonization factors have morphology of fimbriae or pili (14). However, the morphology of CS6 has not so far been defined. CS6 was assumed to be either a nonfimbrial or a short oligomeric assembly that does not protrude enough to be visualized under an electron microscope (17). Functional CS6 is expressed and transported to the bacterial surface in a chaperone-usher pathway. CssC and CssD are the chaperone and usher proteins, respectively, that help surface expression of the CS6 structural subunits,CssA and CssB (33).

The role of CS6 in intestinal adherence has been demonstrated using CS6-expressing whole bacteria, but the receptor specificity is still unknown (11). A recent report has shown that when CssB is mutated, binding of bacteria to a colonic cell line (CaCo-2) is reduced slightly compared to that of the bacteria expressing whole CS6 (30).

Here, we have purified CS6 to homogeneity from a clinical isolate of ETEC and separated its subunits (CSSA and CSSB) for the first time. We have characterized CS6 in its native form and demonstrated that fibronectin (Fn) is the interacting matrix for adherence. The carboxy-terminal (C-terminal) region of CSSA plays a key role in this interaction with the amino-terminal (N-terminal) region of Fn.

MATERIALS AND METHODS

Bacterial isolate and growth conditions. ETEC isolate 4266 (serogroup O167, LT+) expressing CS6 as the only CFA (7) was used in this study. This strain was isolated from a patient with diarrhea undergoing treatment at the Infectious Diseases Hospital, Kolkata, India. For expression of CS6, the strain was grown overnight in CFA broth (1% Casamino Acids, 0.15% yeast extract, 0.05% MgSO₄, 0.005% MnCl₂, pH 7.4) (3) and maintained at ~70°C as a 15% glycerol stock. A single colony grown on MacConkey agar (Difco, Detroit, MI) plate at 37°C was subcultured in CFA medium for further studies.

Purification of CS6. CS6 was purified from the ETEC 4266 strain by chromatographic methods using a DuoFlow system (Bio-Rad, Hercules, CA). ETEC 4266 was grown overnight at 37°C in CFA broth, and heat-saline extract was...
preparing as described earlier (15). Briefly, the bacterial cells were incubated in 0.8% NaCl at 60°C for 20 min with mild shaking (20 rpm), and the supernatant was collected by centrifugation at 8,000 × g for 20 min. The heat-saline extract was then subjected to ammonium sulfate precipitation, and the pellet from 40 to 60% ammonium sulfate saturation was dissolved in buffer A (20 mM Tris-HCl, pH 7.5). The protein sample (~3 mg) was dialyzed overnight in the same buffer and loaded onto a 3-ml Q-Sepharose FF column (GE Healthcare, Uppsala, Sweden) previously equilibrated in buffer A. The protein bound to the anion-exchange matrix was eluted with a linear NaCl gradient of 0 to 0.6 M NaCl at a flow rate of 1 ml/min. C6, along with minor contaminating proteins, were eluted at approximately 0.4 M NaCl in buffer A. The fractions containing C6 were pooled and dialyzed in buffer A overnight at 4°C. The dialyzed sample was concentrated on a 1-ml UNO Q1 column (Bio-Rad) by step elution to 0.6 M NaCl in buffer A. Peak fractions were loaded onto a Superdex 200 (GE Healthcare) gel filtration column (1 cm by 40 cm) previously equilibrated with 0.15 M NaCl in buffer A at a flow rate of 0.5 ml/min. The C6-containing pooled fractions were again concentrated using the UNO Q1 column as described before. Purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after each step. The identities of the purified proteins were confirmed by N-terminal protein sequencing. The protein concentration was determined with the Bradford protein estimation kit (Bio-Rad) in all cases. The absence of lipopolysaccharide in the final preparation was monitored by estimation of neutral sugar (2).

Cloning and sequencing ofCssA and CssB. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) from an overnight culture of ETEC grown in CFA broth at 37°C, and cDNA was prepared using a reverse transcription kit (Promega, Madison, WI) following manufacturer’s protocol. For PCR amplification, the cDNA was amplified based on the previous study (accession numbers U04846 and U04844) (33) and were procured commercially (Isogen Biosciences, Germany). The primers used were CsaA (forward) (5’TACACAGAAATGCGCCTAA-3’) and CsaA (reverse) (5’GTGTTTACATAGTAACCAACC-3’) for CsaA and CsbB (forward) (5’AGGAAACGCGACTATATAAT-3’) and CsbB (reverse) (5’ATTGTCGTTAATATGACAG-3’) for CsbB. After initial denaturation of cDNA at 94°C for 3 min, annealing was performed at 55°C for 45 s and extension was done at 72°C for 45 s. After 20 cycles, the PCR product was cloned in pGEM-T Easy cloning vector (Promega) and transformed into competent E. coli (JM109 cells). Positive colonies were selected by blue-white selection in IPTG (isopropyl-β-D-thiogalactopyranoside)/X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactophanopyranoside) plates supplemented with ampicillin. From the correct clones, the respective plasmid was purified using the Wizard Plus DNA purification system (Promega). Using the plasmid as a template, CsaA and CsbB were sequenced with an automated DNA sequencer (ABI Prism 3200; Applied Biosystems, Foster City, CA) using the above-mentioned primers.

Expression and purification of recombinant C6. As for the entire construction of the whole C6 opsonin, primers were designed based on the available sequences as mentioned above. Forward and reverse primers were 5’-AGCCAAATGCGCTGC TTGAGTAAATC-3’ and 5’-CTCAACATTGTTTATTTACAACA-3’ for CsaA and CsbB, respectively. After initial denaturation of cDNA at 94°C for 3 min, annealing was performed at 55°C for 45 s and extension was done at 72°C for 45 s. After 20 cycles, the PCR product was cloned in pETM-Easy (Promega) and transformed in JM109 following the manufacturer’s protocol. Positive colonies were selected by blue-white selection in IPTG (isopropyl-β-D-thiogalactopyranoside)/X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactophanopyranoside) plates supplemented with ampicillin. From the correct clones, the respective plasmid was purified using the Wizard Plus DNA purification system (Promega). Using the plasmid as a template, CsaA and CsbB were sequenced with an automated DNA sequencer (ABI Prism 3200; Applied Biosystems, Foster City, CA) using the above-mentioned primers.

Expression of CsaA and CsbB. Recombinant CsaA and CsbB were expressed in BL21 (DE3) (Stratagene) cells using the pETM-Easy vector system (Novagen, St. Louis, MO) was intraperitoneally injected into mice, followed by two booster doses of C6 with incomplete adjuvant (Sigma). Antibody against the C-terminal peptide of CsaA was prepared as described before (7). The antibody specificity of the antibody was confirmed by Western blotting. Briefly, an equal amount of pure C6 was resolved in 15% SDS-PAGE, and the proteins from the gel were transferred to nitrocellulose membrane (Bio-Rad). To ensure equal loading, the membrane was stained with 0.2% Ponceau S (Sigma) in 0.1% glacial acetic acid. CsaA and CsbB were focused with the respective antibodies using nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside) as the substrate. The absorbance at 492 nm was obtained by measuring the areas under the peaks from the chromatogram. The fatty acid composition of CsaA and CsbB was determined by GLC analysis.

Determination of molecular mass by ESI-MS. The electrospay ionization-mass spectrometry (ESI-MS) analysis was performed by using an API 2000 mass spectrometer (Applied Biosystems). Before analysis, the samples were dialyzed extensively in distilled water and finally brought into 50% acetonitrile with 0.1% acetic acid. The final concentrations of CsaA and CsbB were approximately 11 μM. To determine the molecular mass, positive ESI spectra with m/z values ranging from 650 to 1,800 were studied, and the mass was determined by using Analyst 1.4.1 software (Applied Biosystems). GLC analysis. Purified subunits (2 μM each) were completely saponified with 0.5 N methanolic KOH separately. For determination of fatty acid composition by gas-liquid chromatography (GLC) (6890N; Agilent Technologies, Santa Clara, CA), methyl esters were prepared following standard methods (17). A DB Wax column in a flame ionization detector, the fatty acid composition was analyzed. The percentage composition of component fatty acids was determined by measuring the areas under the peaks from the chromatogram. The fatty acid peaks were identified by comparison with standard methyl esters (Sigma).

Computational analysis. The primary sequences of both CsaA and CsbB were determined from the gene sequence. To design peptides for the binding studies, the crystal structure was analyzed with the program OSTRIC (Oxford University Press Studio) (18). Stretches of surface-exposed, hydrophilic regions were selected as probable binding regions of CsaA. Solid-phase binding assays. To demonstrate matrix adhesion, binding assays were performed with ETEC, pure C6, and peptides. Ninety-six-well ELISA

To demonstrate matrix adhesion, binding assays were performed with ETEC, pure C6, and peptides. Ninety-six-well ELISA
plates were coated with Fn, collagen IV, and laminin (Sigma), whenever necessary, as described earlier (27). ETEC 4266 was biotinylated (25) as a tool for detection and incubated with immobilized Fn for 1 h to determine the adherence. Adhesion was quantitated by ELISA following standard protocols using HRP-conjugated avidin (Sigma). Increasing concentrations of purified CS6 (0 to 80 nM) were used in competition assays against the ETEC strain. The dissociation constant (K_d) for the CS6 binding was determined using increasing concentrations of CS6 (0 to 80 nM), and bound CS6 was determined with anti-CssB polyclonal antibodies (1:500 dilution) followed by HRP-conjugated secondary antibodies using a standard ELISA protocol. The dissociation constant for CS6 with Fn was determined by the equation obtained from the curve-fitting data:

\[
Y = \frac{F_{\text{max}}}{X}(\frac{1}{K_d} + \frac{1}{K_d})
\]

where \(X\) is the molar concentration of CS6 determined. Binding of CS6 to Fn was competed with purified CsaA and CsaB (100 \(\mu\)M from a 60-ng/\(\mu\)l solution of each), and the bound CS6 was detected using anti-CssB antibodies. To define the interacting domain of Fn, the N-terminal 30-kDa fibrin binding domain, 45-kDa gelatin binding domain, and 70-kDa domain were obtained commercially (Sigma). All the domains were dialyzed extensively in 20 mM Tris-HCl (pH 6.8), and equal molar concentrations (26.7 nM) of the Fn domains were applied to ELISA plates. Binding of CS6 with the respective domains was determined by fitted dose-response curves using increasing concentration of CS6 (0 to 500 nM) from the equation:

\[
Y = A[I + (A2X)^{1/2}]
\]

where \(X\), A1, A2, and A3 are the CS6 concentration, F_{max}, association constant, and Hill coefficient, respectively.

**Intrinsic tryptophan fluorescence studies.** For determination of the interaction between CsaA and CsaB, both subunits were dialyzed in buffer A, and CsaB was titrated with CsaA. CsaB contained one tryptophan (at position 3 of the mature protein), but CsaA had no tryptophan. When CsaA was added to CsaB, the binding of CsaA caused quenching of the fluorescence intensity of CsaB. The fluorescence measurements were performed at 25°C on a Hitachi F3010 spectrofluorimeter (Hitachi Ltd., Tokyo, Japan) using an excitation wavelength of 295 nm and recording emission at 340 nm. The excitation and emission band passes were 5 and 10 nm, respectively. The absorbance of the solution was <0.1 absorption unit/cm at the excitation wavelength, and the fluorescence intensity was corrected for dilution when ligand solution was added. For measuring the K_d, we used the following equation (23) obtained from curve fitting:

\[
F/F_0 = (1 + (1 - F_{\text{ref}})^{1/2})^2(K_d + nP_L + L) - (K_d + nP_L + L)^2 - 4nP_LK_d)^{1/2}
\]

In equation 3, \(F_0\) and \(F\) are the fluorescence intensities of CsaB at 340 nm in the absence and in the presence of CsaA, respectively, at a given concentration, \(L\), \(F_{\text{ref}} \) and \(F_{\text{sat}}\) are the observed fluorescence ratios at any given concentration (\(L_p\)) and at the saturating ligand concentration, respectively. The end point of titration (\(F_{\text{ref}}\)) is indicated by no further change in fluorescence intensity upon addition of more ligand, \(P_L\) is the protein concentration, \(K_d\) is the dissociation constant, \(n\) is the stoichiometry, and \(L_p\) is the total ligand concentration.

In order to determine the binding of CsaA and the peptide quenching of tryptophan fluorescence, the intensity of Fn was measured. CsaA or the peptides did not have any tryptophan, so purified CsaA or the peptides were used for monitoring fluorescence quenching of Fn. Increasing concentration of CsaA (3.43 \(\mu\)M stock) and peptide A3 (Peptide A3 (NYTSKGEDKIPPGIYN) (3.12 mM stock) were added in aliquots separately to Fn (900 nM), and the K_d was determined from the following equation (29) as obtained from the curve-fitting of the data:

\[
\Delta F(\%) = \Delta F_{\text{ref}}[L]/(K_d + [L])
\]

where \([L]\) is the total concentration of ligand and \(\Delta F\) is the fluorescence intensity change observed at 340 nm. \(\Delta F(\%) = 100(F_{\text{ref}} - F_i)/F_{\text{ref}}\) where \(F_{\text{ref}}\) is the fluorescence intensity at 340 nm of the Fn-ligand complex and \(F_i\) is the fluorescence intensity at 340 nm of Fn.

**Cell culture.** Intestinal cell line INT 407 was grown to confluence in 12-well tissue culture plate in Dulbecco’s modified Eagle’s medium (Sigma) with 10% fetal bovine serum at 37°C with 5% CO_2.

**Confocal microscopy.** CS6-expressing ETEC isolate 4266 was incubated with anti-CsaA antibody (1:200) for 1 h and washed twice at 6,000 \(\times\) g at 4°C by centrifugation. Bacterial cells were incubated with anti-rabbit secondary anti-body tagged with fluorescein isothiocyanate (FITC). Bacterial cells were washed for four times in PBS by centrifugation and visualized under a confocal microscope (LSM 510 Meta; Carl Zeiss, Germany) at a 520-nm emission wavelength and a magnification of 4×0.

To study colonization of Csa6 and Fn, INT 407 cells grown on sterile coverslips were incubated with Csa6 for 1 h. After incubation with Csa6, cells were fixed with 2% paraformaldehyde in PBS (pH 7.2). The cells were incubated with anti-CsaA antibody and focused with FITC-tagged secondary antibody. After being washed twice, cells were further incubated with anti-Fn antibody, followed by secondary antibody tagged with R-phycocerythin (Sigma, St. Louis, MO). After incubation for 1 h, cells were washed by dipping the coverslips in PBS thrice and mounted upside down with glycerol on a glass slide for visualization under a confocal microscope. To study colonization, images were taken at emission wavelengths of 520 and 590 nm for FITC and R-phycocerythin, respectively, and merged images were created.

**Bacterial binding to the intestinal cell line.** An overnight bacterial culture was harvested by centrifugation and resuspended in PBS. CS6-expressing ETEC (~5 × 10^8 CFU) were incubated with a confluent INT 407 monolayer, and the involvement of the CsaA subunit in binding to Fn was compared by preincubating equal amounts of ETEC separately with Fn, anti-CsaA (1:200), and anti-CssB (1:200) antibodies for 1 h. After 3 h of incubation, nonadherent bacteria were removed by washing the cells five times in PBS. Epithelial cells were then detached by treatment with trypsin-EDTA and plated on MacConkey agar (Difco, Detroit, MI) by serial dilution as described earlier (30). The number of the colonies formed on MacConkey agar plates after overnight incubation at 37°C represented the viable bacterial cells that were bound to INT 407 cells.

**FIG. 1.** (A) Purification of CS6. The protein profile of each fraction was monitored by 15% SDS-PAGE and visualized by Coomassie blue staining. Lane 1, crude heat-saline extract; lane 2, 40 to 60% ammonium sulfate cut; lane 3, ion-exchange (Q-Sepharose) fraction; lane 4, gel filtration peak after concentration on a UNO Q1 column. A representative gel picture is presented. (B) Stoichiometry of CS6 subunits. The relative intensities of CsaA and CsaB were analyzed densitometrically from SDS-PAGE followed by Coomassie blue staining. Data are represented graphically as means ± standard errors of the means from six independent experiments, with each well containing varied amount of purified CS6. Inset, representative Coomassie blue-stained SDS-polyacrylamide gel of CS6 containing CsaA and CsaB. (C) CS6 RNA expression analysis. Normalized RT-PCR results are represented graphically. Equal amounts of RT-PCR products were run on a 1% agarose gel and visualized following ethidium bromide staining. Gel images were analyzed densitometrically using parC as an internal control. The fold increases of CsaA and cssB were quantified with respect to the parC band intensity. Each bar represents the mean ± standard error of the mean from three independent experiments. Inset, representative agarose gel showing ethidium bromide-stained, PCR-amplified CsaA, CsaB, and ParC.
FIG. 2. (A) Differential scanning calorimeter studies. Pure CS6 (0.99 μg/μl) dialyzed in PBS was analyzed in a differential scanning calorimeter. The peaks at 70.94°C and 75.41°C represent the transition temperatures of CssB and CssA, respectively (black line). Dashed lines denote the deconvoluted peaks of the resultant peak (black solid). The gray solid line is the fitted line. (B) Purification of CssB. Representative emission spectra of CssB and native CS6 on excitation at 295 nm to monitor refolding of CssB are shown. (C) Purification of CssA. CssA was purified by heating CS6 repetitively at 72°C and monitored by SDS-PAGE and Coomassie blue staining (lanes 1 to 4). Purified CssA is shown in lane 4. M, molecular weight marker. (D) Interaction between CssA and CssB, showing quenching of tryptophan fluorescence emission spectra of CssB by titration with CssA. A representative result from three independent experiments is presented here. The $K_d$ was determined to be 3.2 pM.

Curve fitting. Data fitting was done using Kpyplot (version 2.0, beta 13) (1, 29) to obtain the best-fit curves for $K_d$ determination. The $K_d$ values presented here are the means from at least three separate experiments.

CS6 binding to Fn was calculated from the absorbance reading in ELISA. The $K_d$ was calculated from equation 1, obtained from the best-fitted curve. During binding of CS6 with proteolytic Fn fragments, the maximum absorbance reading was considered the saturation range. We found that the curve was nonlinear, so fitting was done to account for the cooperative binding, and equation 2 was obtained. In case of CssA-CssB interaction, the minimum quenching ratio was considered the saturation range. As we did not know the stoichiometry for detection of the $K_d$ value, fitting parameters (for example, $n$ and $F_{max}$) were varied systematically using the nonlinear least-squares fit procedure to obtain the best-fit curve. We found that an $n$ value of 1 gave the best-fit curve. Binding of CsSA or the peptide to Fn was calculated from fluorescence quenching. The maximum percentage of quenching was considered the saturation range. We found that the curve was nonlinear, so fitting was done to account for the cooperative binding, and equation 2 was obtained to determine the $K_d$ value.

Nucleotide sequence accession numbers. The CssA and CssB sequences of the ETEC 4266 isolate were deposited in GenBank under accession numbers EF451566 and EF451567, respectively.

RESULTS

Characterization of CssA and CssB of native CS6. To characterize CS6 in its native form, the protein was purified from a clinical ETEC isolate by using different chromatographic techniques. The two subunits, CssA and CssB, remained together during all purification steps (Fig. 1A). Despite having differences in the net charge, the subunits could not be separated by ion-exchange chromatography in the presence of ionic (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (CHAPS) or nonionic (Tween 20 and Lubrol PX) detergents. Both subunits were eluted as single sharp peak in ion-exchange chromatography, behaving like a single protein. In SDS-PAGE with Coomassie blue staining, the relative intensities of the

FIG. 3. Analysis of antibodies against CS6 and its subunits. (A) (i) Quantitative analysis of antibodies against CS6 and its subunits, which were raised in mice and analyzed by Western blotting. Pure CS6 was run in SDS-PAGE, transferred to nitrocellulose membrane, and stained reversibly with Ponceau S. The membrane was cut into strips, and each strip was developed with primary antibodies followed by alkaline phosphatase-conjugated secondary antibodies. Lane 1, CS6 focused with anti-CssA antibody (NYTSGDEKKPPGIYN); lane 2, CS6 focused with the anti-CssA antibody; lane 3, CS6 with CssB antibody; lane 4, CS6 incubated with anti-CssB antibody. A representative blot from three independent experiments is presented here. (ii) Ponceau S-stained nitrocellulose membrane before developing with specific antibodies to show CS6 containing equal amount of CssA and CssB in each lane. (B) Determination of anti-CssA antibody titer. Pure CsSA was applied to a 96-well ELISA plate, and anti-CssA antibodies at different dilutions were added in different wells. Ponceau S-stained nitrocellulose membrane before developing with specific antibodies to show CS6 containing equal amount of CssA and CssB in each lane. (C) Determination of anti-CssB antibody titer. Pure CsSB was applied to a 96-well ELISA plate, and anti-CssB antibodies at different dilutions were added in different wells. Each bar represents the mean ± standard error of the mean from three independent experiments. Grey bars denote the antibody titer determined using anti-CssB antibody. White bars denote the antibody titer determined using antipeptide antibody. Black bars denote the antibody titer determined using anti-CS6 antibody. (C) Determination of anti-CssB antibody titer. Pure CsSB was applied to a 96-well ELISA plate, and anti-CssB antibodies at different dilutions were added in different wells. Each bar represents the mean ± standard error of the mean from three independent experiments. Grey bars denote the antibody titer determined using anti-CssB antibody. White bars denote the antibody titer determined using antipeptide antibody. Black bars denote the antibody titer determined using anti-CS6 antibody. Preimmune serum was used as a negative control. Each bar represents the mean ± standard error of the mean from three independent experiments.
subunits as analyzed by densitometric analysis suggested that Cssa and Cssb may be present in equal stoichiometry (Fig. 1B). The relative expression of cssA and cssB analyzed by semiquantitative RT-PCR suggested that equal amounts of transcript were made (Fig. 1C), probably indicating equal production of both subunits.

To understand the thermal stability of CS6, differential scanning calorimetry was used. Purified CS6 gave two characteristic transitions at 70.94°C and 75.41°C (Fig. 2A). SDS-PAGE analysis showed that when pure CS6 was incubated at 70°C, CssB was precipitated and Cssa was left in solution, confirming the transition temperatures for CssB and Cssa as 70.94°C and 75.41°C, respectively. Based on the transition temperatures, Cssa and Cssb were purified (see Materials and Methods for details). The derived amino acid sequence indicated that only CssB, and not Cssa, had one tryptophan residue. Thus, the refolding of CssB was monitored by comparing the tryptophan fluorescence of the native CS6 (Fig. 2B). Pure Cssa was prepared by repeating the heating process several times (Fig. 2C). To determine the Kd for interaction between Cssa and Cssb, Cssb was titrated with Cssa. By quenching of the tryptophan fluorescence intensity of Cssb, the Kd was determined to be $3.2 \times 10^{-12}$ M (Fig. 2D), obtained from equation 3. The binding constant suggests that the interaction between the subunits was virtually irreversible. We found that the data were best fitted using an n value of 1 in equation 3 (n is the stoichiometry), and no significant improvement of the fit was observed when n was >1. This also suggested that Cssa and Cssb bound to each other at a probable stoichiometry of 1:1.

Studies of antibody response against CS6 subunits. To raise antibodies against Cssa and Cssb, the protein bands were crushed from SDS-polyacrylamide gels and injected into mice. Western blotting showed that anti-Cssb antibody was produced but Cssa antiserum was not produced. When CS6 was

<table>
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<tr>
<th>Subunit</th>
<th>Length (amino acids) of mature protein</th>
<th>Molecular mass, kDa (mean ± SEM)</th>
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<tbody>
<tr>
<td>Cssa</td>
<td>136</td>
<td>14.96 ± 0.19, 18.46 ± 0.18, 15.06 ± 0.17</td>
</tr>
<tr>
<td>Cssb</td>
<td>146</td>
<td>16.06 ± 0.18, 15.92 ± 0.2</td>
</tr>
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a Calculated based on the gene sequence by the Expasy proteomic server (http://www.expasy.org/).
b Calculated with respect to standard marker proteins in a gel documentation system from six different SDS-PAGEs.
c Analyzed with the API 2000.
d Mass of native form of Cssa.
e Mass obtained after in-source fragmentation.

FIG. 4. Analysis of modification on CS6. (A) MS analysis of the purified Cssa. Cssa (11 μM) was analyzed by MS, and two peaks were obtained. (B) MS analysis of the purified Cssb. Cssb (11 μM) was analyzed by MS, and one peak was obtained. (C) GLC analysis of Cssa. Methyl ester was prepared from 2 μM of Cssa and analyzed in a DB Wax column in a flame ionization detector. (D) GLC analysis of Cssb. Methyl ester was prepared from 2 μM of Cssb and analyzed in a DB Wax column in a flame ionization detector. (E) Western blotting analysis to monitor any change in the relative mobility of Cssa. Equal amounts of untreated and treated (with methanolic chloroform) CS6 were focused with monospecific anti-Cssa peptide antibody (1:500). A representative blot from two independent experiments is presented here.

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taining both subunits was used for immunization, antiserum against CssB alone was obtained (Fig. 3A). In contrast, when the C-terminal antigenic region was used for immunization in the form of a keyhole limpet hemocyanin-conjugated synthetic peptide (NYTSGDKEIPPGIYN), specific antibody against CssA was obtained, as described earlier (7). Antibody produced against CS6 or its individual subunits indicated that CssA was very poorly immunogenic.

Next, ELISA was performed to analyze the titers of these antibodies (Fig. 3B and C). Antibody raised against whole CS6 interacted with the CssA subunit at a dilution of 1:50 (Fig. 3B). However, the same antibody interacted with CssB at a dilution of 1:1,000 (Fig. 3C). The peptide antibody interacted withCssA at a dilution of 1:1,000, but antiserum raised against whole CssA showed almost no binding in ELISA. The results further confirm thatCssA is poorly immunogenic in mice. However, the presence of CssB could induce a poor immune response againstCssA when they are injected together.

**Molecular mass analysis of CS6 subunits.** The molecular masses of pure CssA and CssB were estimated based on SDS-PAGE and MS (Table 1). In the MS, pure CssA gave two peaks, one at 18,459 Da and the other at 15,063 Da (Fig. 4A). The molecular mass of 15,063 Da corresponded well with the theoretical molecular mass of the CssA subunit. The presence of the other peak at 18,459 Da raised the possibility of modification of CssA. In the case of CssB, however, the molecular mass that was determined by MS (15,924 Da) matched perfectly with its theoretical prediction (Table 1).

In order to identify the modification on CssA, GLC was performed. CssA modification was converted to methyl esters, and when the retention times for the peaks were compared with those for the known standards, palmitic, stearic, and oleic acids were identified (Fig. 4C). The relative amounts of the fatty acids present, as calculated from the areas under the peaks, were 58.4, 28.7, and 12.9% for palmitic, stearic, and oleic acids, respectively. No such modification was observed on CssB (Fig. 4D). When CS6 was treated with methanolic chloroform, the mobility of CssA did not change from that of the other peak at 18,459 Da raised the possibility of modification of CssA. In the case of CssB, however, the molecular mass of 15,063 Da corresponded well with its theoretical prediction (Table 1).

**CS6-expressing ETEC binds to immobilized Fn.** To determine the probable binding substrate, different intestinal extraacellular matrixes (Fn, laminin, and collagen IV) were tested for binding of CS6-expressing ETEC. Among these, Fn was found to be the probable binding matrix (Fig. 5A). CS6-expressing ETEC bound to Fn in a CFU-dependent saturable manner (Fig. 5B). The ETEC binding to Fn could be blocked by competition with purified CS6, suggesting its specific involvement in this interaction (Fig. 5C). Purified CS6 also bound to immobilized Fn in a concentration-dependent, saturable manner (Fig. 5D). The $K_d$ was determined as $77.28 \pm 8.5$ nM from equation 1, suggesting that the binding was strong.

**Identification of theCssA region that binds to Fn.** In order to find out which subunit of CS6 played a role in Fn binding, a preincubation experiment was carried out. Preincubation of Fn with CssA inhibited binding of whole CS6 to this matrix by 24-fold ($P < 0.05$) (Fig. 6A). However, preincubation of Fn with CssB did not affect the binding of CS6, suggesting the specific involvement of CssA in Fn binding. Direct evidence ofCssA-Fn binding was obtained from quenching of the intrinsic fluorescence intensity of Fn on addition of pure CssA. CssA bound Fn in a concentration-dependent, saturable manner. The $K_d$ of interaction was $163 \pm 9.7$ nM (Fig. 6B), as determined using equation 4.

To define the interacting region(s) of the CssA subunit in Fn binding, surface-exposed, flexible regions of the subunits were selected, since they are the most probable regions for binding to a host receptor(s) (Table 2). Based on the analysis, three peptides corresponding to these identified regions were synthesized. The C-terminal region of CssA (residues 112 to 126) (NYTSGDKEIPPGIYN) blocked the binding of CS6 to Fn significantly (79% inhibition, $P < 0.05$) (Fig. 6C). Binding of the peptide to Fn was in a concentration-dependent, saturable manner. The $K_d$ was $23 \pm 3.1$ nM (Fig. 6D) as obtained from equation 4. To confirm whether the C-terminal region of CssA was indeed surface exposed in its native state, a confocal microscopic study was performed. Using the anti-CssA peptide antibody followed by FITC-tagged secondary antibody, live ETEC could be visualized under the microscope, suggesting that the binding region was exposed outwardly (Fig. 6E).

**Identification of theCS6 binding domain in Fn.** To identify the interacting region of Fn involved in binding with CS6, three commercially available proteolytic fragments of human Fn...
were used. Among the three domains tested, the 70-kDa domain showed maximum interaction with CS6 (K_d = 5.8 nM), followed by the fibrin binding 30-kDa domain (K_d = 110 ± 12 nM) and the gelatin binding 45-kDa domain (K_d = 251 ± 31 nM) (Fig. 7A). When competed with the Pep A3, binding of CS6 with the 70-kDa domain decreased in a concentration-dependent manner (Fig. 7B), suggesting that the binding was specific.

Role of fatty acid modification in Fn binding. Western blot analysis showed that recombinant CssA expressed in E. coli HB101 had a higher mobility than the native CssA (Fig. 8A), indicating that recombinant CssA might not have any fatty acid modifications. The molecular mass of recombinantCssA was found to be around 15 kDa in SDS-PAGE, which was similar to the theoretical calculated value based on the number of amino acids. Binding experiments using ELISA suggested that native CssA and recombinant CssA bound to Fn similarly (Fig. 8B).

ETEC binding to INT 407 cells. CS6-expressing ETEC was found to interact with INT 407 cells (Fig. 9A). When purified

Figure 6. Cssa-mediated CS6 binding to Fn. (A) Binding of CS6 (2 μM of CS6 in each well) was competed by prior incubation of Cssa or Cssb (100 μl from a 60-ng/μl solution of each) in ELISA. Each bar represents the mean ± standard error of the mean from four independent experiments (P < 0.05). (B) Intrinsic fluorescence quenching of Fn (0.44 μM) on titration by addition of purified Cssa. The K_d was determined to be 163 nM. A representative graph is presented here. (C) Graphical representation of binding inhibition of CS6 by different peptides on coated Fn by ELISA. Equal molar concentration of peptides were incubated with Fn before addition of CS6. A1, A2, A3, B1, and B2 represent Pep A1, Pep A2, Pep A3, Pep B1, and Pep B2, respectively (see Table 2). Each point is the mean ± standard error of the mean from four independent experiments. (D) Quenching of tryptophan fluorescence emission spectra of Fn (900 nM) by incubation with Pep A3 (NYTSGDKEIPPGIYN) (3.12 mM stock). The binding constant was determined to be approximately 23 nM. A representative result is presented here. (E) Representative picture of ETEC, showing that the peptide region is surface exposed. Live bacteria were stained by incubation with anti-CssA antibody followed by FITC-labeled secondary antibody, mounted on a glass slide, and visualized under a confocal microscope at a magnification of ×40.

Table 2. Inhibition of binding of CS6 to Fn on competition with different regions of Cssa and Cssb

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide used for inhibition</th>
<th>Position</th>
<th>Inhibition of binding to Fn (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cssa</td>
<td>Pep A1: VYPVYDEYYG</td>
<td>57-66</td>
<td>1.32 ± 0.15</td>
<td>0.865</td>
</tr>
<tr>
<td></td>
<td>Pep A2: VDDKGRKMLKDH</td>
<td>85-96</td>
<td>7.95 ± 0.53</td>
<td>0.419</td>
</tr>
<tr>
<td></td>
<td>Pep A3: NYTSGDKEIPPGIYN</td>
<td>112-126</td>
<td>79.47 ± 3.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cssb</td>
<td>Pep B1: NYDSDPDKL</td>
<td>32-39</td>
<td>1.99 ± 0.22</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>Pep B2: VNNPDQMNNYIRKD</td>
<td>78-92</td>
<td>12.58 ± 0.74</td>
<td>0.249</td>
</tr>
</tbody>
</table>

* Peptides were designed based on surface-exposed, flexible stretches of each subunit using the PLOTSTRUCTURE program of Discovery Studio.

* Position in mature protein based on gene sequence.

* Calculated with respect to the control, in which binding of CS6 was monitored. CssB peptides were used a negative control. Data represent means ± standard errors of the means from four independent experiments (P < 0.05).
CS6 was added to the INT 407 cells together with the ETEC. Decreased binding of ETEC was observed. Preincubation of the ETEC with anti-CssB antibody did not alter the adherence to INT 407 cells. However, preincubation with anti-CssA peptide antibody followed by ETEC with anti-CssB antibody did not alter the adherence. Preincubation of CS6 was added to the INT 407 cells together with the ETEC, diminished binding of ETEC was observed. Preincubation of CS6 with different domains of Fn. Equimolar amounts (28.7 nM) of the N-terminal 30-kDa fibrin binding domain (Δ), the 45-kDa gelatin binding domain (Δ), and the 70-kDa domain (Δ) consisting of both the 30-kDa and 45-kDa domains were applied separately on an ELISA plate. Increasing concentrations of CS6 (0 to 600 nM) were incubated with the immobilized domains to determine the dissociation constant of CS6 for each of the domains. Each point represents the mean ± standard error of the mean from three independent experiments. (B) Immobilized 70-kDa Fn (0.1 μg/well) was preincubated with increasing concentrations of peptide (0 to 60 nM), and binding of CS6 (1.5 μM) was monitored. The dissociation constant was determined to be 10 nM. Each point represents the mean ± standard error of the mean from four independent experiments.

FIG. 7. Analysis of Fn domains involved in CS6 binding. (A) Binding of CS6 with different domains of Fn. Equimolar amounts (28.7 nM) of the N-terminal 30-kDa fibrin binding domain (Δ), the 45-kDa gelatin binding domain (Δ), and the 70-kDa domain (Δ) consisting of both the 30-kDa and 45-kDa domains were applied separately on an ELISA plate. Increasing concentrations of CS6 (0 to 600 nM) were incubated with the immobilized domains to determine the dissociation constant of CS6 for each of the domains. Each point represents the mean ± standard error of the mean from three independent experiments.

DISCUSSION
ETEC expresses an array of colonization factors that enables it to colonize the host intestine and cause diarrhea. Although CS6 is one of the most prevalent CFAs, it has not been characterized in detail in terms of structure-function relationships. In this study, we have purified CS6 in its native form from a clinical isolate of ETEC for the first time, characterized it, and elucidated the binding of CS6 with Fn.

During the purification steps, both subunits remained together, behaving like a structural unit. Neither of the subunits has cysteine, so there is no possibility of forming a disulfide linkage between them, suggesting that the interaction between the subunits is noncovalent in nature. Such a strong noncovalent association between two proteins is not common, but it exists in biological systems. The interaction between avidin and biotin, for example, shows association in the femtomolar range (8). The presence of equal amounts of the two subunits in Coomassie blue-stained SDS-polyacrylamide gels suggested that CsaA and CsbB may be present in equal stoichiometry. The relative expression of the subunits in RT-PCR confirmed equal production of the transcripts at the RNA level. The studies of binding between CsaA and CsbB also suggested that the stoichiometry of the subunits is 1:1. Previously, it was suggested that the stoichiometry might be 3:1 based on relative signal intensities from the N-terminal protein sequencing data (33). However, it was also suggested in the same report that the stoichiometry might be 1:1, and our results support the same. This 1:1 stoichiometry is more probable, as the CS6 operon has a region of dyad symmetry at 6 bp downstream of CsbB, and this region essentially acts as a transcription terminator ensuring equal production of both of the subunits (33).
The predicted molecular weight of CssB was little higher than that of CssA, but contrary to the theoretical prediction, CssB migrated faster in SDS-PAGE. MS suggested that CssA had modifications, which accounts for the anomalous migration in SDS-PAGE. GLC results confirmed these modifications in the CssA as palmitic, stearic, and oleic acids. During MS analysis, a portion of these fatty acids onCssA might have undergone aerial oxidation at the source during ionization, giving rise to two species of CssA (modified and unmodified). This modification was covalently attached, since treatment with methanolic chloroform could not remove the fatty acids, ruling out the possibility of any noncovalently attached artifact.

In bacterial systems, such fatty acid modification is not an uncommon phenomenon. For example, hemolysin of *E. coli* (28) and the Fn binding protein BBK32 of *Borrelia burgdorferi* (26) are known to have lipid modifications that help in membrane anchoring. The precise role of the lipid modification on CssA is not yet clear.

Purified CssA is poorly immunogenic in mice, but antibodies at very low titers could be obtained in the presence of CssB. However, antibody could be raised against a synthetic unmodified peptide of CssA. Recently, it has been shown that antibody could be raised against a recombinant CssA in mice (30).

In the present study, we have shown that pure Cs6 binds to Fn of INT 407 intestinal cells. The C-terminal region of CssA mediates the interaction. The low dissociation constant of this interaction indicated that the binding is strong and specific. We have found that Cs6 binds to both the N-terminal 29-kDa and 45-kDa domains of Fn. Together, both domains (which form the 70-kDa domain) bind to Cs6 strongly. It may be possible that the binding motif of Fn for Cs6 may be overlap between

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**FIG. 9.** CS6-mediated ETEC binding to INT 407 cells. (A) Relative binding of ETEC to INT 407 cells. Equal numbers of the bacteria (5 × 10^6 CFU) were used to compare the binding to INT 407 cells. The relative binding capacity was denoted as percentage of bound bacteria per ml of incubated bacteria. The significance level was monitored with respect to the binding of uncompeted ETEC. i, ETEC; ii, ETEC preincubated with Fetuin; iii, ETEC preincubated with Fn; (iv) INT 407 cells were preincubated with Cs6 before addition of ETEC; v, INT 407 cells were preincubated with CssA before addition of ETEC; vi, INT 407 cells were preincubated with CssB before addition of ETEC. (B) Colocalization of Cs6 with Fn in INT 407 cells. (i) Phase-contrast microscopic view of INT 407 cells. (ii) Red, Fn on cell surface labeled with anti-Fn antibody followed by R-phycoerythrin-tagged anti-mouse secondary antibody. (iii) Green, Cs6 labeled with anti-CssB antibody followed by FITC-tagged anti-mouse secondary antibody. (iv) Yellow, merged image suggesting colocalization of Cs6 with Fn. The panels are representative images from two separate experiments viewed at a magnification of ×40.

Previous reports have indicated that several gram-positive and gram-negative bacteria bind to Fn (19, 26). ETEC strain B34289c, isolated from an infantile diarrhea patient, was found to bind Fn. A 17-kDa protein representing a curlin, along with 55-kDa and 29-kDa surface proteins, were presumed to be involved in this interaction with Fn (5, 31). However, no specific receptor for Cs6 has been identified to date. Here, we have clearly demonstrated for the first time that Cs6 binds to Fn. Our results suggest that CsaA is involved in interaction with Fn. In a very recent report, it has been shown that an *E. coli* strain expressing recombinant Cs6 binds to colonic cell line CaCo-2 through Cs6B (30). Our results could not highlight a similar function of Cs6B. The apparent discrepancy in the observations may be due to the difference in cell lines and the use of laboratory *E. coli* expressing recombinant Cs6 instead of pure protein.
29-kDa and 45-kDa domains or that both are involved in CS6 binding in a cooperative manner. When the Fn binding abilities of the recombinant CS6 and native CS6 were compared, the two proteins showed comparable binding to Fn, suggesting that fatty acids may not have a role in binding. Confocal microscopic studies showed that CS6 binds along the cell junctions, where Fn was also located. Co-localization of CS6 and Fn along the cell junction indicated specific interaction between these proteins on the cell surface. Our result suggests that Fn is the major binding matrix for CS6 in INT 407 cells; however, involvement of another receptor cannot be ruled out, as confocal microscopic data revealed the presence of bound CS6 in some other regions of cells. Further studies are required to define this additional receptor(s).

ETEC is mainly a non-invasive pathogen. However, there are reports that intestinal cell membrane asymmetry is altered during ETEC infection (13). The presence of fecal leukocytes (10) and interleukin-8 (9) in stool samples from ETEC-infected patients has been observed. We have also observed an eightfold increase in interleukin-8 secretion upon incubation with CS6 in intestinal cell lines, indicating that CS6 might induce inflammation (unpublished observation). Fn is found to interact with CS6 in intestinal cell lines, indicating that CS6 might serve as a major binding matrix for CS6 in INT 407 cells; however, in vivo microscopic data revealed the presence of bound CS6 in some other regions of cells. Further studies are required to define this additional receptor(s).

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