Two specific amino acid variations in colonization factor CS6 subtypes of enterotoxigenic Escherichia coli results in differential binding and pathogenicity

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CS6 is the predominant colonization factor of enterotoxigenic Escherichia coli (ETEC). We report the existence of multiple CS6 subtypes caused by natural point mutations in cssA and cssB, the structural genes for CS6. The subtype AIIBI was mostly associated with ETEC isolated from diarrhoeal cases, whereas AIIBII was mostly found in asymptomatic controls. Here we explore the rationale behind this association. ETEC isolates expressing AIIBII showed weaker adherence to intestinal epithelial cells compared with ETEC expressing AIIBI. AIIBII expression on the ETEC cell surface was threefold less than AIIBI. We found that alanine at position 37 inCssAII, in conjunction with asparagine at position 97 inCssBII, was responsible for the decreased levels of AIIBII on the bacterial surface. In addition, purified AIIBII showed fourfold less mucin binding compared with AIIBI. The asparagine at position 97 inCssBII was also accountable for the decreased mucin binding by AIIBII. Reduced fluid accumulation and colonization occurred during infection with ETEC expressing AIIBII in animal models. Together these results indicate that the differential adherence between AIIBI and AIIBII was a cumulative effect of decreased surface-level expression and mucin binding of AIIBII due to two specific amino acid variations. As a consequence, ETEC expressing these two subtypes displayed differential pathogenicity. We speculate that this might explain the subjective association of AIIBI with ETEC from diarrhoeal cases and AIIBII with asymptomatic controls.

INTRODUCTION

Among Escherichia coli pathotypes, enterotoxigenic E. coli (ETEC) is the most common cause of diarrhoeal illness in children under 5 years of age, adults in the developing world and in travellers to these areas (Black, 1990, 1993). Annually, ETEC is estimated to cause 200 million diarrhoeal episodes during epidemic or sporadic outbreaks and approximately 380 000 deaths (WHO, 2006).

The pathogenicity of ETEC depends on the presence of several virulence factors such as heat-labile and heat-stable enterotoxins, as well as colonization factors (CFs). To date, more than 25 CFs have been identified in ETEC (Gaastra & Svennerholm, 1996; Qadri et al., 2005). Colonization is mediated by the interaction of CFs with their cognate receptors on the intestinal epithelium to enable the bacteria to withstand intestinal peristalsis and initiate the infection. This is followed by release of enterotoxins, thereby initiating pathogenesis.

CS6 is a predominant CF which is present in approximately 30% of ETEC isolated globally (Wolf, 1997). The CS6 operon, cssABCD, is composed of four ORFs encoding the two major structural subunitsCssA and CssB, whereas sequence homology suggests that CssC andCssD are periplasmic chaperone and molecular usher, respectively (Wolf et al., 1997). The entire sequence of the plasmid harbouring CS6 has been determined (Wajima et al., 2013). Unlike most other CFs, CS6 is non-fimbrial and, in a sense, its subunit composition and assembly are also unique. CS6 is composed of the two heterologous structural subunits CssA and CssB to form a tight protomer in equal stoichiometry (Gastra & Svennerholm, 1996; Wolf et al., 1997; Ghosal et al., 2009). A recent structural study showed that the CS6 protomer forms oligomers (Roy et al., 2012). Intestinal...
colonization by ETEC strains expressing CS6 involves interactions with fibronectin and sulpholipid (Ghosal et al., 2009; Jansson et al., 2009). In addition, CS6 could also bind to rabbit intestinal mucin (Helander et al., 1997).

CsaA and CssB from different ETEC isolates display several natural mutations in their respective structural genes, giving rise to allelic variants (Wolf et al., 1997; Sabui et al., 2010). We have designated these allelic variants AI, AII, AIII, BI and BII, which gave rise to five subtypes of CS6 reported thus far (Sabui et al., 2010). Of these subtypes, there is preferential association of AIBI with clinical isolates of ETEC, and AIIBII with asymptomatic cases of ETEC infection (Sabui et al., 2012).

In the present study, we investigated the association of AIBI and AIIBII subtypes with the symptomatic and asymptomatic phenotypes of ETEC isolates. ETEC strains expressing these two CS6 subtypes showed differential cellular binding (Sabui et al., 2010). As a CF, two factors can conceivably affect the functional efficacy of CS6: the level of expression on the ETEC surface and the intrinsic affinity of CS6 to host epithelial cell surface receptors. Accordingly, we compared the presentation of AIBI and AIIBII on the ETEC surface and their binding to mucin. We found that AIIBII has reduced surface expression as well as decreased affinity for mucin. These two factors cumulatively contributed to significant reduction in the colonization efficiency of AIIBII subtypes, in comparison with that of AIBI. As a consequence, we established an altered level of pathogenesis in animal models during infection by ETEC isolates expressing these two CS6 subtypes. Results presented here provide a plausible explanation for the preferential association of ETEC expressing the AIBI and AIIBII subtypes with diarrhoeal cases and asymptomatic controls, respectively.

METHODS

Bacterial strains and growth conditions. ETEC isolates 3023 and 4266 (LT+, ST+) isolated from diarrhoeal patients admitted to the Infectious Diseases Hospital, Kolkata, India (Ghosal et al., 2007), having similar background but expressing different CS6 subtypes, were used in this study. For purification of AIBI and AIIBII subtypes of CS6 from ETEC 4266 and 3023 strains, respectively, overnight bacterial cultures in CFA broth (1% Casamino acids, 0.15% yeast extract, 0.05% MgSO4, 0.0005% MnCl2, pH 7.4) at 37 °C were used. E. coli XL1-Blue, JM109 (Promega) was used for plasmid construction and maintenance. E. coli BL21 (DE3) (Promega) was used for expression of recombinant proteins. These laboratory strains were grown in Luria–Bertani medium (BD Difco), with appropriate antibiotics where needed, at 37 °C.

Construction of isogenic CS6 mutants and CS6 complement vectors. ETEC 4266 expressing AIBI was used as the parental strain for construction of isogenic mutants of CS6 of AcsAI and AcssBI as described previously (Wajima et al., 2011). Plasmids pcssAIBI and pcssAIIIBI were constructed by amplifying a region from 1.5 kb upstream of cssA to 1.5 kb downstream of cssD, using CS6-F and CS6-R as primers (Table S1, available in the online Supplementary Material). The 7.1 kb CS6 product was digested with Sall (TaKaRa Bio) and cloned into the Sall site of pSTV28 (TaKaRa Bio). The expression plasmids pcssAI/pcssBI and pcssAII/pcssBII were constructed by inverse PCR using pcssAIBI and pcssBI as template, respectively. pcssAI/pcssAII was used to complement ΔcssAI whereas pcssBII/pcssBIII was used for the complementation of ΔcssBI.

Cell culture. The human intestinal cell line HT-29 was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 1% (v/v) non-essential amino acids and 1% (v/v) penicillin-streptomycin solution (Sigma) at 37 °C in a 5% CO2 incubator (Mondal et al., 2014).

Adhesion assay. For detection of bound bacteria by ELISA, mid-exponential phase bacterial culture was used for biotinylation as described previously (Sabui et al., 2010). Different concentrations of biotinylated bacterial suspensions (100 μl per well) were added to 96-well plates coated with a monolayer of HT-29 epithelial cells and then incubated for 3 h. After washing, the cell monolayer was fixed for 10 min at room temperature (RT) with 50 μl of 4% paraformaldehyde and adhesion was detected by horseradish peroxidase (HRP)-conjugated avidin (1:800) using 3,3,5,5’-tetramethylbenzidine (BD OptEIA) as a substrate at 450 nm. A bacterial suspension of 107 c.f.u. ml−1 was pelleted down and dissolved in the culture medium. This suspension was added to epithelial cells grown on 12-well plates at 80–90% confluence. After 3 h of incubation at 37 °C in 5% CO2, cells were washed three times with PBS and detached using 0.1% Triton X-100. Adherent bacteria were counted after serial dilution by plating on MacConkey agar (BD Difco) plates.

For immunofluorescence, HT-29 cell monolayers were cultured in 24-well polystyrene plates containing glass coverslips until 70–80% confluence, and incubated with GFP-labelled bacteria (105 c.f.u. ml−1) for 3 h. The bound bacteria were fixed with 2% formalin after washing three times with PBS and permeabilized with 0.2% Triton X-100 for 10 min at RT, followed by three washes with PBS. A 1:5000 dilution of DAPI (Sigma) in PBS was used for DNA staining and the coverslips were mounted on glass slides with 1% p-phenylenediamine mounting medium. Samples were visualized using an Axio Imager M1 microscope (Zeiss).

Purification of CS6 from ETEC strains. CS6 was purified from heat saline extracts of ETEC strains by chromatographic methods using a Duo Flow system (Bio-Rad) as described previously (Ghosal et al., 2007).

Determination of expression of CS6 on the ETEC surface. A mid-exponential phase bacterial suspension of 107 c.f.u. was incubated with 0.15% SDS followed by washing with ethanol and heated at 100 °C for 5 min. This treated bacterial pellet was then dissolved in 100 μl carbonate buffer (pH 8.2) and was used for coating the ELISA plate to quantify total CS6. To quantify surface expression, we used 107 c.f.u. of ETEC for coating and incubated the bacteria overnight at 4 °C (Elder et al., 1982). Unbound bacteria were decanted and wells were washed three times with PBS followed by blocking in 5% non-fat skimmed milk in PBS. After washing, the bound fraction was determined using anti-CSβ polyclonal antibody (1:300) as primary antibody followed by HRP-conjugated secondary antibody (1:1000). The amount of protein was determined using a standard curve obtained with purified CS6. For the standard curve, different concentrations of CS6 were coated on each well in triplicate and the bound CS6 protein was detected by anti-CSβ antibody (Fig. S1).

For determination of CS6 surface expression by SDS-PAGE, equal numbers of bacteria were subjected to heat saline extraction (Ghosal et al., 2007). The heat saline extract was separated on 15 % SDS-PAGE gels. Gels were transferred to nitrocellulose for Western blot analysis. Blots were blocked in 5% non-fat skimmed milk in Tris buffer saline plus 0.1 % Tween-20 (TBS-T), and incubated in anti-CSβ primary
CS6 subtypes show differential binding and pathogenicity

Antibody solution overnight at 4 °C and an HRP-conjugated isotype-appropriate secondary antibody for 30 min at RT. Blots were developed using HRP-dependent chemiluminescence and visualized by exposure to X-ray film. The intensities of specific bands were measured using the UVP GelDoc software analyser to measure protein expression levels.

Mucin purification from rabbit intestine. Mucin was extracted from intestinal scrapings of rabbits as described by Mondal et al. (2014). The amount of carbohydrate and protein was determined using anthrone reaction (Trevelyan et al., 1952) and the modified Folin–Lowry method (Lowry et al., 1951), respectively.

Solid phase binding assay. To demonstrate receptor–ligand interaction, 96-well Maxisorp ELISA plates (Nunc) were coated with rabbit mucin dissolved in bicarbonate buffer (pH 9.8) at 4 °C overnight. The dissociation constant (Kd) for CS6 binding with mucin was determined by incubating increasing concentrations of CS6 (0–1500 nM) for 3 h at RT, and the bound fraction was determined using anti-CS6 polyclonal antibody (1:300) as the primary antibody followed by HRP-conjugated secondary antibody (1:1000). The Kd for the CS6–mucin interaction was determined from a fitted dose–response curve in the KyPlot program (version 2.0) using increasing concentrations of CS6 from the equation:

\[ Y = \frac{Y_{\text{max}} \times A}{A + K_d} \]

where A is the molar concentration of CS6 determined.

Inhibition of bacterial binding to mucin. One hundred nanograms of rabbit mucin were coated on the wells of a microtitre plate as described above. The wells were then blocked with 5% non-fat skimmed milk in PBS for 2 h. Different concentrations of CS6 purified either from wild-type or complemented strains were applied to the wells in triplicate. After 1.5 h of incubation, unbound proteins were washed with PBS and 1000 c.f.u. of the biotinylated ETEC strain expressing AIBI was added to each well. After 1.5 h of incubation, unbound bacteria were washed with PBS, and HRP-conjugated avidin (1:250 in PBS) was applied to each well and developed as described above.

Amino acid substitution by site-directed mutagenesis. CssAI and CssAlI variants differ from each other by 11 aa whereas CssBI and CssBII differ from each other by 5 aa (Fig. S2). We replaced the amino acids in pcssAII/pcssBI with the respective amino acids of pcssAlI/pcssBII by site-directed mutagenesis and then complemented either in ETEC AcssAI or ΔcssBII. Site-specific mutations were performed using Stratagene’s QuikChange Site-Directed Mutagenesis kit (Agilent Technologies) as per the manufacturer’s protocol. Mutagenic primers (Table S1) were designed using Stratagene’s web-based QuikChange Primer Design Program.

Adherence and pathogenesis in animal model. The fluid accumulation (FA) assay was done in suckling mice and rabbits. Three- to 4-day-old Swiss mice were intragastrically inoculated with 10⁶ c.f.u. of the appropriate ETEC strain. After 18 h, the mice were weighed and their intestines were removed. Each of the separated intestines was weighed, and the FA ratios were calculated as described by Baselski et al. (1977). PBS-fed mice was used as negative controls. Finally, the intestine was homogenized in 1 ml of PBS (taken as crude). Adhered bacteria were counted by plating on MacConkey agar. The experiments involved four or five newborn mice for each bacterial strain.

For rabbit ileal loop experiments, 6-week-old New Zealand rabbits (male, weighing 1–2 kg) were fasted 48 h prior to the experiment and surgery was carried out under anaesthesia. The general anaesthetic ketamine-50 [35 mg (kg body weight)⁻¹] and local anaesthetic xylcocaine 2% (5 mg kg⁻¹) were administered intravenously. Intestine was taken out through a mid-line incision in the abdomen and intestinal loops of 10 cm were made with 2 cm interloops on both sides. To each loop, 10⁵ c.f.u. of bacterial cells was added and PBS was used as the negative control. FA was measured after 18 h of incubation and adherent bacteria were counted by homogenizing the intestinal section in 1 ml PBS. All animal experiments were conducted following the guidelines of the Institutional Animal Ethical Committee [Apro/77/24/11/2010, reg. no. NICE/CPCSEA (AW) 215/2009-2015].

Statistical analysis. All data were analysed by one-way ANOVA software. A probability level (P) value of ≤0.05 was considered statistically significant. Three independent experiments were done in triplicate and the data represent mean ± SEM of these independent events.

RESULTS

ETEC-expressing CS6 subtypes show differential binding with intestinal epithelial cells in vitro

To define the functional role of CS6 subtypes, adherence with cultured epithelial cells was determined by ELISA. The binding assay showed that ETEC isolates expressing either AIBI or AIIIBII bind to epithelial cells in a c.f.u.-dependent, saturable manner (Fig. 1a). Interestingly, ETEC isolates expressing AIBI bind in greater numbers than ETEC expressing AIIIBII. The data showed ETEC strains expressing AIBI and AIIIBII adhered at 98×10⁵ and 11×10⁵ c.f.u. ml⁻¹, respectively. To quantify the binding difference between two CS6 subtypes with HT-29 cells, we used a c.f.u. counting method. AIIIBII-mediated binding to epithelial cells was ninefold lower (Fig. 1b) than AIBI-mediated cellular binding. Furthermore, this difference in adhesion was supported by fluorescence microscopy studies. The number of GFP-labelled ETEC expressing AIIIBII bound to epithelial cells was significantly less than ETEC expressing AIBI (Fig. 1c). The difference in binding between ETEC expressing CS6 subtypes to epithelial cells in vitro was statistically significant (P<0.05).

ETEC expressing AIIIBII subtype showed reduced colonization in animal models

Colonization was studied in a suckling mouse model. ETEC isolates expressing AIBI and AIIIBII adhered at 51×10⁵ and 6×10⁵ c.f.u. g⁻¹, respectively. The number of c.f.u. (per gram of intestine) recovered from each mouse after infection with ETEC expressing AIIIBII was eightfold lower than ETEC expressing AIBI (Fig. 2a). To confirm these results, a rabbit model was used in which the number of bound bacteria was ninefold (P<0.05) less in the case of AIIIBII-ETEC (Fig. 2b).

CssBII is responsible for reduced cellular binding and colonization

Next, we performed a binding assay with the HT-29 intestinal cell line using a ΔcssAI/ΔcssBII mutant and


ΔcssAI: cssAI, ΔcssAI: cssAII, ΔcssBI: cssBI and ΔcssBI: cssBII complemented ETEC mutants (Fig. 3a). The cellular binding ability of ΔcssAI and ΔcssBI mutants was drastically reduced ($P<0.05$) compared with the wild-type isolate. When the ΔcssAI strain was complemented with pcssAI (ΔcssAI: cssAI), the strain showed binding levels similar to wild-type ETEC expressing AIBI. Similar results were found for the ΔcssAI: cssAII ETEC strain. Furthermore, when the ΔcssBI strain was complemented with pcssBI, the binding was similar to the wild-type strain. However, upon complementation with pcssBII, the binding could not be reversed efficiently. The CssBII complemented ΔcssBI mutant showed eightfold less binding than the wild-type AIBI-expressing strain. This suggests that CssBII is responsible for the reduced binding of the ETEC expressing the AIBI subtype.

We confirmed the in vitro binding data in the suckling mouse model using isogenic mutants and complemented ETEC strains. The CssAI, CssAII and CssBI complemented ETEC strains showed binding similar to that of wild-type ETEC expressing AIBI, with the exception of the CssBI complemented strain. The CssBII complemented strain showed eightfold less binding (Fig. 3b).

**Fig. 1.** Differential binding of ETEC strains expressing AIBI and AIIBII. (a) Different concentrations of biotinylated ETEC expressing AIBI and AIIBII were allowed to interact with HT-29 in a 96-well plate. Each bar represents mean ± SEM of three independent experiments. (b) In total, $10^7$ c.f.u. of ETEC expressing AIBI and AIIBII were incubated with HT-29 intestinal cells for 3 h and adherent bacteria were determined by the plate counting method. Each bar represents mean ± SEM of three independent experiments. In all binding assays we used an isogenic mutant as background. *$P<0.05$. (c) Inverted fluorescence micrographs of CS6 subtypes showing mediated ETEC adherence to HT-29 cells. (i) Non-infected control cells; (ii) cells infected with ETEC expressing AIBI; (iii) cells infected with ETEC expressing AIIBII. Green fluorescence corresponds to GFP expressed by ETEC strains. DNA in HT-29 cells was stained blue with DAPI.

ETEC expressing the AIIBII subtype show reduced expression of CS6 on the bacterial surface

We tested for CS6 expression on the surface of ETEC isolates expressing either AIBI or AIIBII (Fig. 4). There was a threefold difference in surface expression of AIBI and AIIBII subtypes ($P<0.05$) (Fig. 4a), although the total amount of CS6 subtypes produced in these ETEC strains was similar. The RNA expression of each subunit of these strains was also comparable (Fig. S3).

The role of CssA and CssB subunits in the expression of CS6 on the ETEC surface was determined using ΔcssAI: cssAI, ΔcssAI: cssAII, ΔcssBI: cssBI and ΔcssBI: cssBII complemented ETEC strains. When the ΔcssAI strain was complemented with pcssAI (ΔcssAI: cssAI), surface expression of CS6 remained similar to that of the wild-type ETEC expressing AIBI (Fig. 4b). Similar results were obtained with the ΔcssBI: cssBI complemented strain. However, the CssAII complemented strain (ΔcssAI: cssAII) showed a 1.7-fold reduction in expression level and the CssBII complemented strain showed 2.5-fold reduction compared with ETEC expressing AIBI (Table S2). This indicated that CssAII and CssBII are responsible for reduced surface expression of AIIBII CS6. Similar results were found when surface
expression was analysed densitometrically by measuring the intensity of protein bands from wild-type and complemented strains (Fig. 4b).

**Gly<sup>39</sup> in CssAI and Lys<sup>97</sup> in CssBI play an important role in CS6 expression on ETEC surface**

CssAI and CssAII variants differ from each other by 11 aa (Fig. S2a). To determine the key amino acid residues of the CssA subunit responsible for altering the surface expression, we used site-directed mutagenesis to replace these 11 aa residues of CssAI with the corresponding amino acid residues in CssAII at the same position. These plasmids were used to complement ETEC ΔcssAI. Of 11 point mutations in CssAI only the replacement of Gly<sup>39</sup>Ala led to a 1.6-fold reduction in surface expression of CS6 (Fig. 5).

CssBI and CssBII differ from each other by 5 aa (Fig. S2b). Similarly, we determined the key amino acid residues of the CssB subunit responsible for differential surface expression. Lys<sup>97</sup>Asn in CssBI resulted in a 2.8-fold reduction in surface expression of CS6 (Fig. 5). The results indicated that Gly<sup>39</sup> in CssAI and Lys<sup>97</sup> in CssBI play an important role in surface expression of CS6.

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**Fig. 2.** In vivo intestinal colonization assay. (a) In vivo colonization studies using ETEC strains expressing AIBI and AIBII subtypes. Mice were orally inoculated with 10<sup>7</sup> c.f.u. and the degree of ETEC colonization was measured after 18 h as c.f.u. (g of intestine)<sup>−1</sup>. Each bar represents mean±SEM of at least four independent experiments. (b) Intestinal colonization was done in rabbit ileal loop. Each 10 cm loop was inoculated with 10<sup>7</sup> c.f.u. of ETEC strains expressing AIBI and AIBII. At 18 h of infection, the rabbit was euthanized and two or three 2 cm sections from each loop were homogenized in 1 ml of PBS, then ETEC adherence was measured (c.f.u. g<sup>−1</sup>). In all colonization assays we used the isogenic mutant as background. *P<0.05.

**Fig. 3.** Cellular binding of wild-type, its isogenic mutants and complemented ETEC strains. (a) In total, 10<sup>7</sup> c.f.u. of AIBI-ETEC, ΔcssAI, ΔcssAI:cssAI, ΔcssAI:cssAII, ΔcssBI, ΔcssBI:cssBI and ΔcssBI:cssBII were used for binding studies. Each bar represents mean±SEM of three independent experiments. Each bar indicates mean±SEM of at least three independent experiments. An asterisk represents a statistically significant (P<0.05) difference in binding.
AIBI and AIIBII display differential mucin binding

The purified AIBI and AIIBII showed a difference in binding to rabbit mucin. AIBI and AIIBII bound to immobilized rabbit mucin in a concentration-dependent and saturable manner, with $K_d$ values of 312 and 1.256 $\mu$M, respectively (Fig. 6a). AIIBII showed fourfold lower binding to mucin compared with AIBI.

To get a better understanding of the subunit involved in mucin binding, we used purified CS6 subtypes expressed in complemented ETEC strains. AIIBII CS6 purified from ΔcssBII:cssBII ETEC bound to mucin with a $K_d$ of 1.38 $\mu$M. However, the AIIBI CS6 purified from ΔcssAI:cssAI ETEC showed similar binding to mucin as AIBI CS6 purified from either ΔcssAI:cssAI ETEC or ΔcssBI:cssBI ETEC.
This result suggests that differential mucin binding is due to allelic variations in the CssB subunit.

Next, we carried out a competitive binding assay with different CS6 subtypes. Increasing concentrations of AIBI were added to mucin-coated wells and the binding of ETEC expressing AIBI was determined. A gradual decrease in bacterial binding was observed with increasing concentrations of AIBI (Fig. 6c). Similar inhibition was observed when AIIBI was used in competition. However, when either AIIBII or AIBII was used, no competitive inhibition was noted. This observation indicated that CssBII bound less to immobilized mucin compared with CssBI.

Asn97 in the CssBII subunit of AIIBII CS6 is responsible for decreased mucin binding

To determine the key amino acid residues in CssBI involved in mucin binding, we replaced the five aa residues of CssBI individually with the corresponding amino acid residues present in CssBII at the same position (Fig. S4). These five mutants were expressed in the ΔcssB ETEC mutant and were purified for the mucin binding assay. The results showed that replacement of lysine position 97 in CssBI by asparagine caused a threefold reduction in mucin binding compared with AIBI CS6 from the wild-type strain (Fig. S4). There was no significant change in mucin binding for Asn40Glu, Val60Ala, Ser107Pro and His138Asn substitutions in CssBI with respect to the wild-type CS6.

ETEC expressing the AIIBII subtype showed reduced FA

Pathogenesis was studied by measuring intestinal FA in a mouse model (Fig. 8). The FA ratio was three- to fourfold lower in ETEC expressing AIIBII compared with AIBI.
We observed that ΔcssAI: cssAI, ΔcssAI: cssAll and ΔcssBI: cssBl complemented strains with high intestinal binding ability showed more FA whereas the ΔcssAI, ΔcssBI mutants and ΔcssBI: cssBlI complemented strains showed reduced FA ratio because of their low intestinal binding.

**DISCUSSION**

In this study we aimed to understand the reason behind the differential binding of AIBI and AIIBII subtypes of CS6 expressed by ETEC, as well as their respective associations. Our previous observations suggested that ETEC isolates expressing AIBI had stronger adherence ability to intestinal cells compared with ETEC expressing AIIBII (Sabui et al., 2010). Here, we observed that the differential binding between these two ETEC isolates is specific but cell type independent (data not shown). The difference in binding was similar when BL21 expressing these two CS6 subtypes was used for adherence assays (Fig. S5). This further excludes the contribution of other adhesion factors. A similar variability in adherence to HEp-2 cells was observed with the allelic variants of FimH adhesin from Salmonella enterica serovar Typhimurium (Boddicker et al., 2002).

SL1344 and LB5010, two isolates of S. enterica serovar Typhimurium, showed a difference in their ability to adhere to HEp-2 cells. LB5010 (an LT2 derivative strain) bound in large numbers to HEp-2 cells due to its increased ability to form biofilm as compared with the SL1344 bacterial strain.

Our results indicated that the differential adherence had more than one explanation. We found variations in the level of surface expression in CS6 subtypes, and observed higher expression of AIBI on the ETEC surface compared with AIIBII although the total amount of CS6 produced was similar (Fig. 8a). Similar results were obtained when FA was measured in a rabbit model (Fig. 8b). The data suggested that lower intestinal colonization of ETEC expressing AIIBII correlated with its reduced pathogenesis. RNA expression of the elt and est genes was similar in these strains (Fig. S3).

That the presence of the CssBI allele in combination with either CssAI or CssAII is responsible for reduced pathogenesis was further confirmed by studying FA in suckling mice using complemented ETEC strains (Fig. 8c).

*We observed that ΔcssAI: cssAI, ΔcssAI: cssAll and ΔcssBI: cssBlI complemented strains with high intestinal binding ability showed more FA whereas the ΔcssAI, ΔcssBI mutants and ΔcssBI: cssBlI complemented strains showed reduced FA ratio because of their low intestinal binding.*
by each ETEC was similar. Based on the model of CS6 biogenesis (Wolf et al., 1997; Tobias et al., 2008; Wajima et al., 2011), we speculate that amino acid variations at positions 39 and 97 of CssAI and CssBII, respectively, altered the interaction with either of the assembly proteins CssC and CssD, or both, and therefore translocation efficiency was reduced. As a consequence, surface expression of the AIBII subtype was also reduced.

Previous studies showed that CS6-expressing ETEC strains bind to intestinal epithelial cell lines using multiple receptors (Helander et al., 1997; Ghosal et al., 2009; Jansson et al., 2009). Fibronectin and mucin were reported as receptors for CS6 (Helander et al., 1997; Ghosal et al., 2009; Roy et al., 2012). Here we studied the binding of AIBI and AIBBI with mucin and fibronectin. Both AIBI and AIBBI showed similar binding with fibronectin (Fig. S6). However, when we tested binding for mucin, AIBBI had less binding ability for mucin compared with AIBI. Similar observations were reported by Grange et al. (2002), where porcine fimbrial antigen K88 (F4) of ETEC showed the existence of three variants, K88ab, K88ac and K88ad, that bound to the pSTF receptor but with variation in binding ability. Likewise, E. coli type I fimbriae show functional heterogeneity due to minor sequence variations in the fimH gene (Sokurenko et al., 1994, 1995). These sequence variations resulted in quantitative differences in mannose-specific adhesion due to structural differences in the FimH adhesin. The differential binding of AIBI and AIBBI with mucin was facilitated through CssB. This was also confirmed by using recombinant (r) CssBI and CssBII and determining their Ka during binding with mucin. rCssBII showed higher binding with mucin than rCssBI (Fig. S7).

A single amino acid variation may cause a marked change in receptor binding by a protein. We found that lysine 97 of CssBII played a major role in the mucin binding of AIBI during ETEC adherence. Binding was reduced when lysine was substituted by asparagine in CssBII. A single point mutation in FimH variants of Salmonella gallinarum resulted in loss of ability to mediate mannose-sensitive adhesion (Kisiela et al., 2005). The R181 residue of CfaE tip protein plays a critical role in the receptor-binding feature on colonization factor antigen (CFA) I fimbriae of an ETEC (Baker et al., 2009).

The adherence ability of ETEC isolates expressing AIBI and AIBBI subtypes correlated well with pathogenesis in mouse models. ETEC expressing AIBBI showed lower colonization and FA than the AIBI-expressing strain. Altogether, we conclude that the decreased surface levels and mucin binding synergistically determined the reduced adherence ability of ETEC expressing AIBII when compared with ETEC expressing AIBI. Our studies have demonstrated the importance of investigating the role of naturally occurring mutations as these mutations are likely to make a significant contribution to the ETEC expressing CS6-mediated virulence. This information could be helpful in developing therapeutic inhibitors or antibodies, as well as a subunit vaccine, by targeting the AIBI subtype of CS6 against CS6-expressing ETEC infection.

ACKNOWLEDGEMENTS

This investigation was supported in part by grants from the Program of Funding Research Centre for Emerging and Re-emerging Infectious Diseases, Ministry of Education, Culture, Sports, Science and Technology of Japan and National Centre for Global Health and Medicine (24-110), Japan, and Indian Council of Medical Research, Government of India. A. D. was supported by a fellowship (no. DBT-JRF/10-11/129) from Department of Biotechnology, Government of India.

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Edited by: K. Fields