The role of colonization factors CFA/I and CS21 in ETEC pathogenesis using the human enteroid model

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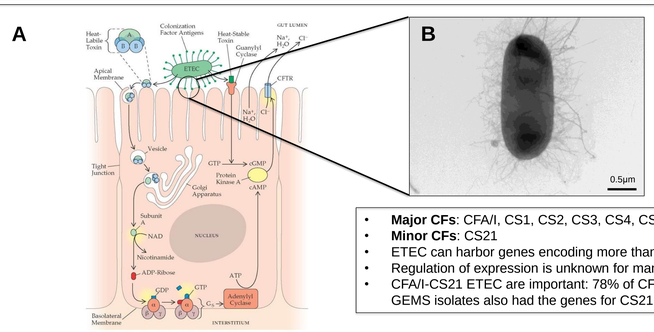
Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is a primary causative agent of diarrhea in travelers and young children in low-to-middle-income countries (LMICs). ETEC adhere to intestinal epithelia via colonization factors (CFs) and secrete heat-stable toxin (ST) and/or heat-labile toxin (LT), causing dysregulated cellular ion transport and water secretion. ETEC isolates often harbor genes encoding more than one CF that are prime targets as vaccine antigens. CFA/I is a major CF that is associated with ETEC isolates that cause moderate-to-severe diarrhea and has been shown to play an important role in ETEC pathogenesis in *in vitro* and *in vivo* studies. The Global Enteric Multicenter Study finding that 78% of CFA/I-expressing ETEC also encode the minor CF CS21 prompted investigation of the potential combined role of these two CFs. Western blots and electron microscopy demonstrated growth media-dependent and clinical isolate-dependent differences in CFA/I and CS21 expression. The critical role of CFA/I, but not CS21, in adherence was demonstrated using the human enteroid model and a series of CFA/I- and CS21-specific mutants, suggesting an alternative role for CS21 in ETEC pathogenesis. Furthermore, only anti-CFA/I antibodies inhibited adherence by global ETEC isolates expressing CFA/I and CS21. Delivery of ST and resulting cGMP secretion was measured in apical and basolateral supernatants from infected enteroid monolayers, and strain-specific ST delivery and time-dependent cGMP production was observed. Interestingly, cGMP levels were similar across wildtype and CF-deficient strains, reflecting the limitations of this static infection model that lacks flow and stretch and is exclusively aerobic. Despite adherence by ETEC and delivery of ST, the enteroid monolayer integrity was not disrupted, as shown by the increase in transepithelial resistance and the lack of inflammatory cytokines produced during infection. Altogether, these data demonstrate that targeting CFA/I in global clinical CFA/I-CS21 strains is sufficient for adherence inhibition, supporting a vaccine strategy that focuses on blocking major CFs. In addition, the enteroid model has significant utility for the study of ETEC pathogenesis.

ETEC Pathogenesis & Role of Colonization Factors



- **Major CFs**: CFA/I, CS1, CS2, CS3, CS4, CS5, CS6
- **Minor CFs**: CS21
- ETEC can harbor genes encoding more than one CF
- Regulation of expression is unknown for many CFs
- CFA/I-CS21 ETEC are important: 78% of CFA/I+ GEMS isolates also had the genes for CS21

Figure 1. (A) Model showing intestinal pathogenesis of ETEC (Madhavan *et al*, 2015). (B) Expression of CFA/I fimbriae by ETEC H10407 examined using transmission electron microscopy.

Human Enteroid Model

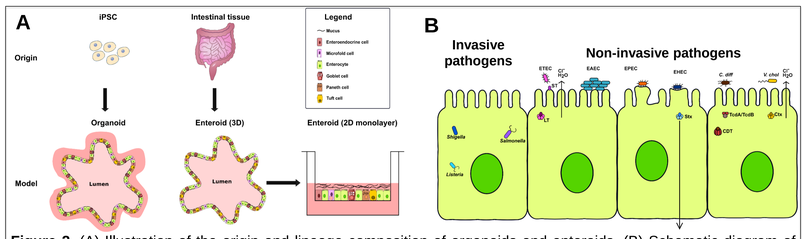


Figure 2. (A) Illustration of the origin and lineage composition of organoids and enteroids. (B) Schematic diagram of the molecular pathogenesis mechanisms of invasive and noninvasive enteric bacteria in this system (Ranganathan SR, Smith EM *et al*, 2020).

ETEC Colonization Factor Protein Expression

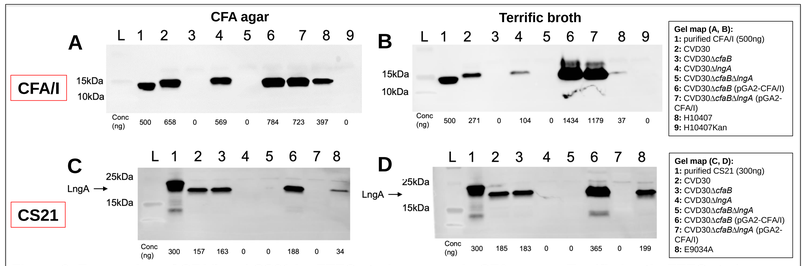


Figure 3. Expression of CFA/I and CS21 by ETEC strains grown in CFA agar or Terrific broth. ETEC strains were grown on CFA agar (A and C) or in Terrific broth overnight static culture (B and D). Whole cell bacterial lysates were probed with anti-CFA/I (A and B) or anti-CS21 antibodies (C and D). Expected band size for CFA/I is 15kDa. Expected band size for CS21 is 22 kDa.

ETEC CFA/I & CS21 Expression & Function

CF Surface Expression

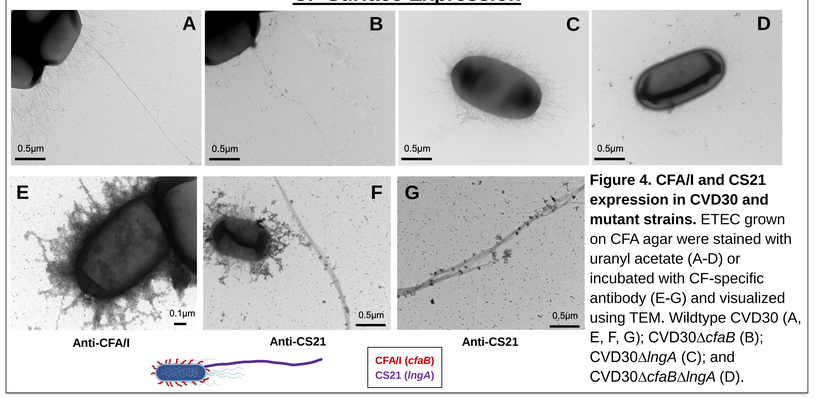


Figure 4. CFA/I and CS21 expression in CVD30 and mutant strains. ETEC grown on CFA agar were stained with uranyl acetate (A-D) or incubated with CF-specific antibody (E-G) and visualized using TEM. Wildtype CVD30 (A, E, F, G); CVD30Δ*cfaB* (B); CVD30Δ*lngA* (C); and CVD30Δ*cfaB*Δ*lngA* (D).

Confocal Microscopy of CVD30 Adherence to Human Ileal Enteroids

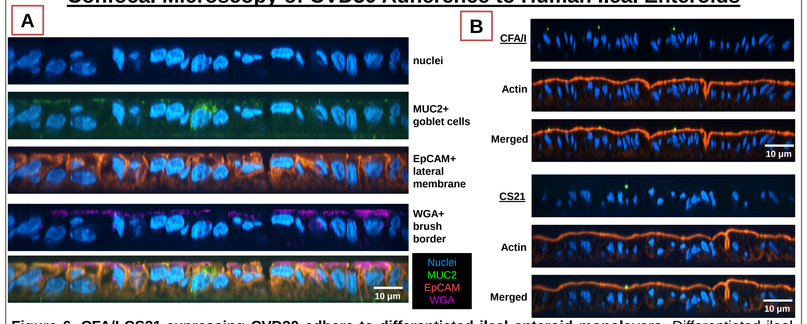


Figure 6. CFA/I-CS21-expressing CVD30 adhere to differentiated ileal enteroid monolayers. Differentiated ileal enteroid monolayers were uninfected (A) or infected with ETEC CVD30 grown on CFA agar (B). Enteroids were washed with PBS and fixed for confocal microscopy. Uninfected monolayers were stained for specific intestinal cell markers (A) and infected monolayers were stained for CFA/I or CS21 (B). Confocal images are in the *xz* plane.

Investigation of CF-Mediated Adherence & ST Delivery to Enteroid Monolayers

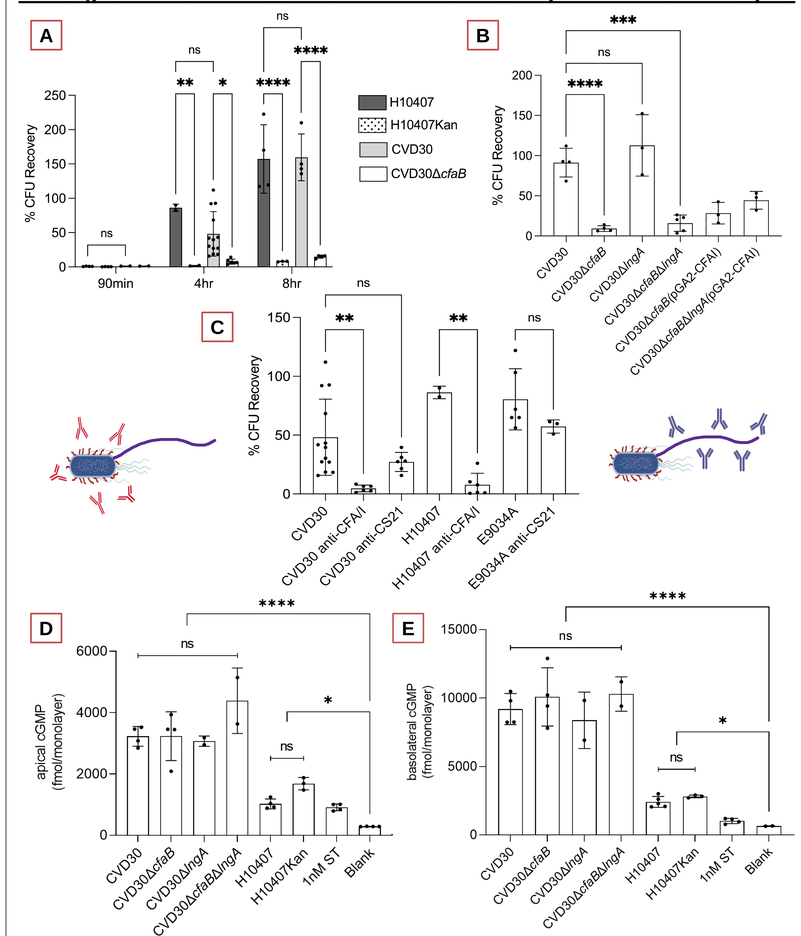


Figure 5. CFA/I alone is important for adherence by ETEC with multiple CFs, while CF-mediated ST delivery was not observed. Differentiated ileal enteroid monolayers were infected with wildtype ETEC strains, CF-deficient mutant strains, or strains pre-incubated with CF-specific antibodies for 4hrs (B, C) or up to 8hrs (A). Enteroids were washed to collect adherent bacteria (% CFU recovery). Enteroid monolayers were infected with wildtype ETEC or mutant strains or purified ST for up to 8hrs and resulting apical and basolateral cGMP production was measured by cGMP ELISA (D, E). Statistical significance was measured using ANOVA tests: * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

Enteroid Barrier Integrity Following ETEC Infection

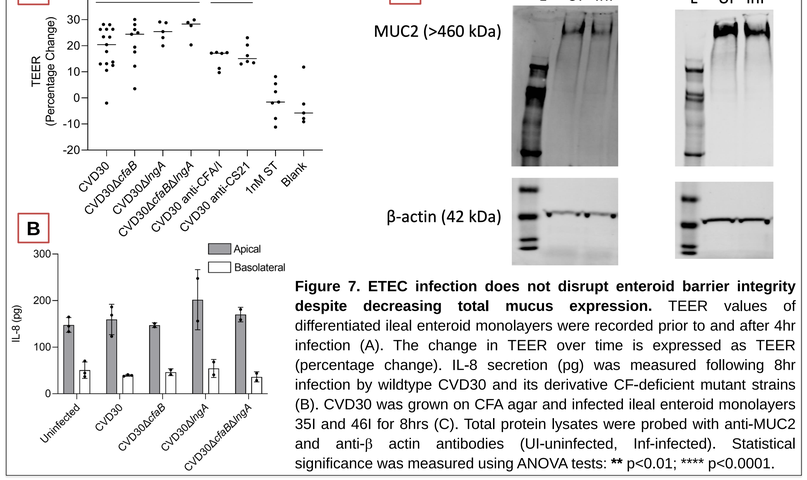


Figure 7. ETEC infection does not disrupt enteroid barrier integrity despite decreasing total mucus expression. TEER values of differentiated ileal enteroid monolayers were recorded prior to and after 4hr infection (A). The change in TEER over time is expressed as TEER (percentage change). IL-8 secretion (pg) was measured following 8hr infection by wildtype CVD30 and its derivative CF-deficient mutant strains (B). CVD30 was grown on CFA agar and infected ileal enteroid monolayers 35I and 46I for 8hrs (C). Total protein lysates were probed with anti-MUC2 and anti-β actin antibodies (UI-uninfected, Inf-infected). Statistical significance was measured using ANOVA tests: ** p<0.01; **** p<0.0001.

Conclusions

- **Clinical ETEC isolate CVD30 express CFA/I and CS21 simultaneously when grown on CFA agar and in Terrific broth. Differential CF expression is observed by growth condition and by strain.**
- **The human ileal enteroid model expresses specific intestinal cell subtypes and serves as a valuable model to study ETEC pathogenesis.**
- **CVD30 express CFA/I and CS21 during infection of enteroid monolayers. CFA/I is important for adherence by ETEC to ileal enteroid monolayers while CS21 is not, based on the use of CF-deficient mutants and strains pre-incubated with CF-specific antibodies.**
- **Delivery of ST and cGMP production is strain specific and increases over time. CF-mediated toxin delivery was not observed, which may be due to limitations of our static, aerobic enteroid model.**
- **ETEC infection does not disrupt enteroid barrier integrity, which is in contrast to host responses to other enteric inflammatory pathogens including *Shigella* and EAEC.**
- **These data support a vaccine strategy that focuses on targeting major CFs, especially CFA/I, to decrease the burden of ETEC-mediated morbidity and mortality globally.**

References

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