Published in final edited form as:

Protein Expr Purif. 2016 March ; 119: 130–135. doi:10.1016/j.pep.2015.11.007.

Simple method for purification of enterotoxigenic *E. coli* fimbriae

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Abstract

Enterotoxigenic E. coli (ETEC) are endemic pathogens in the developing world. They frequently cause illness in travelers, and are among the most prevalent causes of diarrheal disease in children. Pathogenic ETEC strains employ fimbriae as adhesion factors to bind the luminal surface of the intestinal epithelium and establish infection. Accordingly, there is marked interest in immunoprophylactic strategies targeting fimbriae to protect against ETEC infections. Multiple strategies have been reported for purification of ETEC fimbriae, however none is ideal. Purification has typically involved the use of highly virulent wild-type strains. We report here a simple and improved method to purify ETEC fimbriae, which was applied to obtain two different Class 5 fimbriae types of clinical relevance (CFA/I and CS4) expressed recombinantly in *E. coli* production strains. Following removal from cells by shearing, fimbriae proteins were purified by orthogonal purification steps employing ultracentrifugation, precipitation, and ion-exchange membrane chromatography. Purified fimbriae demonstrated the anticipated size and morphology by electron microscopy analysis, contained negligible levels of residual host cell proteins, nucleic acid, and endotoxin, and were recognized by convalescent human anti-sera.

Keywords

Purification; fimbriae; *E. coli*

INTRODUCTION

Gastroenteritis and diarrhea due to infection with ETEC remains a major problem in the developing world, as well as in travelers, including military personnel, from developed nations to areas where these pathogens are endemic [1–5]. The recently completed Global Enteric Multicenter Study (GEMS) revealed that ETEC infections were also among the most common causes of moderate to severe diarrhea in children in the developing world [1].

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Among the virulence factors encoded by ETEC pathogens are fimbriae that mediate bacterial attachment to the gastrointestinal lumen surface, and enterotoxins that cause fluid efflux from the intestinal epithelium resulting in the clinical diarrheal symptoms. The requirement for fimbriae to cause disease is well established; ETEC strains lacking fimbriae are avirulent in humans [6, 7]. Data support the protective capacity of anti-fimbrial responses against challenge with ETEC bearing homologous structures [8, 9]. Accordingly, there is broad interest in understanding their role in the induction of protective immune responses to ETEC, and whether they can serve as the basis of putative ETEC vaccines or as targets of therapeutic antibodies [9–12].

Class 5 fimbriae constitute the largest genetically related group of human ETEC colonization factors and include CFA/I and CS4 [13–15]. Morphologically, ETEC Class 5 fimbriae can be seen as elongated hair-like protrusions extending from the cell surface [11, 12]. The stalk region forms the bulk of the fimbrial structure and is comprised by a multimer of a single major fimbrial protein. A monomer or multimer of the minor fimbrial tip adhesin protein is present at the membrane distal end of the fimbriae and serves as the attachment factor to the intestinal epithelium [10, 16]. Formation of cell-associated fimbriae requires a series of accessory chaperones that mediate transport and assembly at the outer membrane [17]. There are multiple recognized fimbrial types that are each defined serologically by differences within the amino acid sequence of the structural proteins. Pathogenic ETEC generally express one or more distinct fimbriae types [17].

Despite broad interest in the study of the role of ETEC fimbriae in pathogenesis as well as their use as vaccines, the published methods for their production and purification are not optimal. Fimbriae are generally obtained directly from wild-type ETEC cells grown on specialized solid agar nutrient media that is required for optimal induction of fimbrial expression [18]. Fimbriae are detached by mechanical shearing in liquid cell suspensions, followed by removal of insoluble material from the shear supernatant by low-speed centrifugation [19]. De-fimbriation by mechanical shearing results in concomitant release of high levels of protein, nucleic acid and endotoxin impurities [18]. There are several reported strategies for biochemical purification of sheared ETEC fimbriae from shear preparation supernatants. One method made use of sequential fractionation by 2-step differential ultracentrifugation, size-exclusion chromatography and density ultracentrifugation [18, 19]. Alternate methods have employed ammonium sulfate precipitation, followed by fractionation of the precipitated material by size exclusion chromatography or negative chromatography through a weak anion exchange resin [20–22]. Neither of these prior reported methods is ideal. Purification by size exclusion chromatography can be challenging to scale-up, and may not remove impurities that are of similar size or are bound non-specifically to the product. Furthermore, an ion-exchange capture step would be advantageous for fractionation of contaminants that demonstrate both higher and lower sorbent binding affinity compared to the product. We report here an improved method for purification of ETEC fimbriae, applied to two different ETEC fimbriae types expressed constitutively from plasmids in non-pathogenic *E. coli* expression strains.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Cloning of the minimal fimbrial operon of CS4 and CFA/I into plasmid vectors, has been described [11, 23]. Briefly, the CS4 operon was isolated from a genomic library from ETEC strain E11881A and cloned in the expression vector pGA2 (described in [11]). The operon includes 4 genes required for fimbrial expression and is included on a ~5-kb DNA fragment. Expression was driven by the OmpC promoter [11]. The CFA/I operon includes the 4 genes necessary for fimbrial expression that are contained within a ~5-kb operon originally isolated from a genomic library from ETEC strain H10407 [24]. The operon was cloned into plasmid pGA2 as described [23] and is driven by the OmpC promoter. *E. coli* strain DH5 α transformants with these plasmids were used for fimbrial protein expression. Recombinant expression strains were grown in Hy-soy rich media supplemented with 50 μ g/ml kanamycin. Preparation of cultures for fimbrial purification was accomplished as described below. Briefly, a 50 ml liquid starter culture was inoculated with 3–5 colonies from solid agar media plates, and grown for 12 – 18 hours at 37 °C with agitation at 80 rpm. This culture was then used to inoculate a 500 ml culture that was grown under equivalent conditions for 6 – 8 hours. A 4 L fermentation culture in a Biostat A-plus fermenter (Sartorius, Germany) was then inoculated to an OD_{600 nm} of 0.15 with the 500 ml shake flask culture, and maintained 12 – 18 hours at 80 rpm, 5 LPM ambient air, and adjustment to pH 7 using 28 % ammonium hydroxide.

Biochemical purification of fimbriae proteins

Bacteria were harvested from liquid growth cultures by centrifugation at $8,000 \times G$ for 30 minutes at 4 °C in an RC-5B centrifuge (Sorvall) and GS3 fixed angle rotor, and suspended on ice in 1/100th culture volume of phosphate buffered saline (PBS) pH 7.4. Fimbriae were sheared from the suspended culture at 4 °C in a Waring blender with two 30 second pulses at speed 3 and two 30 second pulses at speed 4, leaving a 30 second interval between each pulse. Cells and debris post shearing were removed by $10,000 \times G$ centrifugation for 30 minutes at 4 °C. The resulting supernatant was then subjected to $39,000 \times G$ ultracentrifugation using a Beckman LE-80 Ultracentrifuge with a SW41Ti rotor for 90 minutes at 4 °C. The $39,000 \times G$ supernatant was brought to 1.1 M ammonium sulfate (~15% w/v) with 3.8 M ammonium sulfate solution, incubated overnight at 4 °C, and then centrifuged at $10,000 \times G$ for 30 minutes at 4 °C in an RC-5B centrifuge with a SS34 rotor. The ammonium sulfate pellet was subsequently resuspended in 2 ml of 40 mM Tris pH 7, and dialyzed in 2 kDa molecular weight cutoff dialysis cassettes (Thermo-Pierce) against 2,000 resuspension volumes of 40 mM Tris pH 7 at room temperature for 2 hours, and then overnight at 4 °C after transfer to 2,000 resuspension volumes of fresh buffer. Following dialysis, fimbriae preparations were then loaded onto 3 Sartobind Nano-Q anion-exchange membranes (Sartorius, Germany) (3 ml column volume [CV] each) linked in series at 5 ml/min in 40 mM Tris pH 7 using an AKTA Purifier (GE-Amersham, NJ), with monitoring of UV absorbance at 280 nm and 252 nm. Bound material was washed with 5 CV of binding buffer followed by elution of fimbriae proteins with 5 CV of 40 mM Tris/100 mM NaCl pH 7. Full elution was accomplished with 5 CV of 40 mM Tris/1M NaCl pH 7 to detect fractionated impurities.

SDS-PAGE, Coomassie and Western blot

Samples were prepared by boiling in Laemmli loading buffer (Biorad, CA) and separated by SDS-PAGE on 4–20% acrylamide Tris-Glycine gels (Life Technologies, CA). Visualization of proteins was accomplished by staining with the Gel-code blue Coomassie reagent (Pierce). Analysis for identity by western blot was accomplished by transfer to LF-PVDF membranes (Biorad, CA) and incubation with sera from guinea pigs immunized with a live attenuated *Shigella flexneri* 2a strain expressing ETEC CFA/I or CS4 [23]. Bound antibodies were detected by incubation with goat anti-guinea pig IgG labeled with horse radish peroxidase (KPL, Gaithersburg, MD), and visualized by addition of ECL reagent (Biorad, CA). Densitometry was performed with Quantity One™ software (Biorad, CA).

High performance size-exclusion liquid chromatography (HPLC-SEC)

Analyses were conducted with a Biosep SEC4000 column (Phenomenex, CA) with PBS pH 7.4 as the mobile phase, run at 1 ml/minute on an Alliance 2795 (Waters, MA), and detection of absorbance in fractionated material at 280 nm and 252 nm with a 2489 dual-UV detector (Waters, MA).

Analytical assays

Assessment of endotoxin levels was accomplished by endpoint Limulus Amebocyte Lysate assay with the Endosafe PTS system (Charles River, Mass.). Protein concentration in the final purified material was determined by absorbance at 280 nm using the extinction coefficient of the respective major fimbrial protein calculated using the ProtParam online tool (<http://web.expasy.org/protparam/>).

ELISA

Immulon II ELISA plates (Thermo Scientific, Waltham, MA) were coated with CFA/I (5 or 10 µg/ml) diluted in PBS pH 7.4 for 3 h at 37°C. Plates were washed with PBS Tween (PBST) and blocked overnight with PBS containing 10% dry milk (Nestle, Solon, OH). Pooled serum from human volunteers challenged with ETEC strain H10407 (O78:H11 LT⁺ ST⁺ CFA/I) were added in serial dilutions in PBST with 10% dry milk and incubated for 1 h at 37°C. IgG antibodies were detected with horseradish peroxidase (HRP)-labeled goat anti-human IgG followed by 3,3',5,5'-tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD). The reaction was stopped by adding 1 M H₃PO₄ and plates were read using an Ascent microplate reader (Thermo Scientific, Waltham, MA). The endpoint titer (EU/ml) was calculated through linear regression curves as the inverse of the serum dilution that produces an optical density at 450 nm (OD₄₅₀) of 0.2 above the mean of the blanks.

Electron microscopy

Analyses were conducted at the Core Imaging Facility of the University of Maryland, Baltimore. Fimbriae preparations were applied to glow-discharged, formvar coated copper grids and negatively stained with 1% uranyl acetate for 1 min, air dried and viewed in a Tecnai T12 transmission electron microscope (FEI, Co) at an operating voltage of 80 kV. The images were acquired with AMT XR60B digital camera (Advanced Microscopy Techniques) by using AMTV600 software.

RESULTS AND DISCUSSION

Fermentation growth and harvest of fimbriae

CS4 and CFA/I fimbriae are characterized by ~ 15 kDa major fimbrial proteins CsaB and CfaB, and ~ 40 kDa minor fimbrial proteins CsaE or CfaE respectively. *E. coli* production strains constitutively expressing the entire fimbrial operons of CS4 and CFA/I from plasmids were grown in liquid media. This strategy has several advantages compared to the use of wild-type ETEC strains. Notably, the process is compatible with conventional liquid culture apparatus and methods, does not require specialized media to induce fimbriae expression, and does not carry the occupational safety risks that accompany growth of a virulent human pathogen. Growth was conducted under conditions of low agitation at 80 rpm in order to avoid loss of fimbriae from the cell surface due to shear force. *E. coli* cells expressing CFA/I or CS4 reliably grew to an OD_{600 nm} of ~ 5 after 12 – 18 hours of fermentation growth, with no discernable accumulation of fimbriae evident in the fermentation culture supernatant (Figure 1A, lane 2). Following harvest by low-speed centrifugation, cells were suspended in PBS and fimbriae were removed from the cell mass by mechanical shearing. The sheared cells were then removed by low-speed centrifugation. As is shown in Figure 1, a prominent band at ~ 15 kDa was evident by SDS-PAGE in the sheared supernatant material from CS4 expressing *E. coli*, as well as measurable levels of both higher and lower molecular weight protein bands (Figure 1A, lane 3). Comparable results were found for CFA/I. Fimbriae were subsequently purified from the cleared supernatants using sequential orthogonal biochemical purification steps incorporating separation by size, solubility and charge.

Ultracentrifugation

Bulk insoluble contaminants were first removed by ultracentrifugation at $39,000 \times G$. This step resulted in removal of a measurable level of insoluble contaminants that was evident by a readily discernable pellet. The ~15 kDa CS4 major fimbrial protein remained in the supernatant (Figure 1A, lanes 3–5). Equivalent results were noted for CFA/I (not shown). Although fimbriae are generally very large, we found that significant amounts of the CFA/I fimbrial proteins remained in the supernatant after ultracentrifugation at $\sim 190,000 \times G$ (data not shown).

Ammonium sulfate precipitation

We found that the major subunit of CFA/I fimbriae precipitated maximally with ammonium sulfate concentrations of 15% or greater (Figure 1B). Precipitation with 15% ammonium sulfate similarly precipitated the major fimbrial protein of CS4, whereas many of the other protein contaminants remained in solution (Figure 1A, lanes 6,7). The prior referenced methods that employed ammonium sulfate to precipitate fimbriae used conditions of 40 – 60% saturation [20–22]. Selective precipitation from contaminants at the minimal level of saturation is preferable however, as protein contaminants that remain soluble are readily removed in the supernatant.

Anion exchange chromatography

Conventional ion-exchange resins generally do not allow for efficient capture of large molecules or complexes due to poor accessibility to the porous bead interior. Bacterial flagella share many morphological and structure features with fimbriae, and we have documented previously that they are more efficiently captured by macroporous ion-exchange membranes compared to conventional ion-exchange resins [25]. We found that CS4 fimbrial proteins were captured fully by strong quaternary ammonium ion-exchange membranes (Figure 2). The bound fimbriae proteins were eluted with 100 mM NaCl. The remaining bound impurities were detected after elution with 1M NaCl that matched closely the level and diversity of protein contaminants seen in the starting material (Figure 2A). A lower ratio of A280 nm/A260 nm was seen for the 1M NaCl eluate relative to the 100 mM NaCl eluate, indicating removal of nucleic acid as well as protein (Figure 2B). Equivalent binding and elution profiles were noted for CFA/I fimbriae. A small amount of the major fimbrial protein subunit was detected in the 1 M NaCl elution of both CS4 and CFA/I fimbriae preparations (confirmed by western blot, data not shown). The observed difference in retention could possibly be due to indirect binding to a nucleic acid contaminant, where the affinity may be higher than for the quaternary ammonium sorbent.

Analysis of purified fimbriae preparations

The final protein yield for CFA/I and CS4 fimbriae was on average 3–5 mg of protein per liter of culture. SDS-PAGE and Coomassie analysis of the CFA/I and CS4 fimbriae preparations revealed the presence of a major ~ 12 – 15 kDa band that is at the expected molecular weight of the major fimbrial subunit, and a very faint band at ~ 40 kDa that is consistent with the molecular weight of the minor fimbrial tip protein (Figure 3 A,B). No other major protein bands were evident. The ~ 15 kDa band in CFA/I and CS4 preparations were also recognized by type specific polyclonal anti-sera from guinea pigs (Figure 3C,D). The preparations contained less than 20 endotoxin units per milligram of protein, and low levels of nucleic acid indicated by the high ratio of absorbance at 280 nm relative to 260 nm (Figure 2B, ratio = 1.7). HPLC-SEC analyses revealed that the purified CFA/I proteins fractionated in the column void volume, exceeding the maximal column fractionation limit of ~ 1,500 kDa (Figure 4A). Analysis of the CFA/I and CS4 fimbriae preparations by negative stain electron microscopy further demonstrated the presence of long thin tubes greater than 100 nm in length, consistent with the expected morphology of bacteria associated fimbriae (Figure 4B,C). Taken together, this would indicate that the purified proteins are present almost entirely as large fimbriae multimers. In order to confirm reactivity with sera induced by natural ETEC infection, the purified CFA/I fimbriae preparation was assessed by ELISA with convalescent immune sera obtained from human volunteers infected with ETEC strain H10407 that expresses CFA/I [26]. As detailed in Figure 5, CFA/I fimbrial proteins prepared by this method were recognized by antibodies present in the sera from human adult volunteers infected orally with ETEC strain H10407, which expresses this fimbriae type.

CONCLUSIONS

Recombinant fimbriae expression in conventional *E. coli* production strains is preferable to prior strategies that made use of highly virulent wild-type organisms. This approach permits the use of conventional upstream bacterial growth strategies in liquid media without the need for specialized product expression, and presents minimal occupational health and safety risk. The method for fimbriae purification reported herein provides further advantages over prior approaches. The use of orthogonal purification steps employing different biochemical principles permits robust product separation from protein, nucleic acid and endotoxin contaminants. The methods described are also readily scalable, and optimized for increased yield. Ion-exchange membranes have demonstrated efficient binding properties for a variety of very large macromolecule types (e.g., viral particles, flagella), and are hence well-suited to bind fimbriae [25]. To our knowledge, this is the first report of fimbriae capture and separation by ion-exchange chromatography. There are at least 25 recognized types of ETEC fimbriae. We report an equivalent purification performance for two different representative and clinically relevant ETEC Class 5 fimbriae types, and have found that this strategy is also functional for CS5 fimbriae (data not shown). Fimbriae are virulence factors for a multitude of heterologous bacterial pathogens (e.g., uropathogenic *E. coli*, *Klebsiella pneumoniae*) [27, 28]. This biochemical purification strategy could be applicable to fimbriae from other bacterial species.

References

1. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acacio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet*. 2013; 382:209–222. [PubMed: 23680352]
2. Black RE. Epidemiology of travelers' diarrhea and relative importance of various pathogens. *Rev Infect Dis*. 1990; 12(Suppl 1):S73–79. [PubMed: 2406860]
3. Nada RA, Armstrong A, Shaheen HI, Nakhla I, Sanders JW, Riddle MS, Young S, Sebeny P. Phenotypic and genotypic characterization of enterotoxigenic *Escherichia coli* isolated from U.S. military personnel participating in Operation Bright Star, Egypt, from 2005 to 2009. *Diagn Microbiol Infect Dis*. 2013; 76:272–277. [PubMed: 23639795]
4. Monteville MR, Riddle MS, Baht U, Putnam SD, Frenck RW, Brooks K, Moustafa M, Bland J, Sanders JW. Incidence, etiology, and impact of diarrhea among deployed US military personnel in support of Operation Iraqi Freedom and Operation Enduring Freedom. *Am J Trop Med Hyg*. 2006; 75:762–767. [PubMed: 17038708]
5. Northey G, Evans MR, Sarvotham TS, Thomas DR, Howard TJ. Sentinel surveillance for travellers' diarrhoea in primary care. *BMC Infect Dis*. 2007; 7:126. [PubMed: 17986342]
6. Evans DG, Satterwhite TK, Evans DJ Jr, DuPont HL. Differences in serological responses and excretion patterns of volunteers challenged with enterotoxigenic *Escherichia coli* with and without the colonization factor antigen. *Infect Immun*. 1978; 19:883–888. [PubMed: 346488]
7. Satterwhite TK, Evans DG, DuPont HL, Evans DJ Jr. Role of *Escherichia coli* colonisation factor antigen in acute diarrhoea. *Lancet*. 1978; 2:181–184. [PubMed: 78384]
8. Evans DG, Evans DJ Jr, Opekun AR, Graham DY. Non-replicating oral whole cell vaccine protective against enterotoxigenic *Escherichia coli* (ETEC) diarrhea: stimulation of anti-CFA (CFA/I) and anti-enterotoxin (anti-LT) intestinal IgA and protection against challenge with ETEC

- belonging to heterologous serotypes. FEMS microbiology immunology. 1988; 1:117–125. [PubMed: 3078739]
9. Freedman DJ, Tacket CO, Delehanty A, Maneval DR, Nataro J, Crabb JH. Milk immunoglobulin with specific activity against purified colonization factor antigens can protect against oral challenge with enterotoxigenic *Escherichia coli*. J Infect Dis. 1998; 177:662–667. [PubMed: 9498445]
 10. Poole ST, McVeigh AL, Anantha RP, Lee LH, Akay YM, Pontzer EA, Scott DA, Bullitt E, Savarino SJ. Donor strand complementation governs intersubunit interaction of fimbriae of the alternate chaperone pathway. Mol Microbiol. 2007; 63:1372–1384. [PubMed: 17302815]
 11. Altboum Z, Levine MM, Galen JE, Barry EM. Genetic characterization and immunogenicity of coli surface antigen 4 from enterotoxigenic *Escherichia coli* when it is expressed in a *Shigella* live-vector strain. Infect Immun. 2003; 71:1352–1360. [PubMed: 12595452]
 12. Koprowski H 2nd, Levine MM, Anderson RJ, Losonsky G, Pizza M, Barry EM. Attenuated *Shigella flexneri* 2a vaccine strain CVD 1204 expressing colonization factor antigen I and mutant heat-labile enterotoxin of enterotoxigenic *Escherichia coli*. Infect Immun. 2000; 68:4884–4892. [PubMed: 10948101]
 13. Anantha RP, McVeigh AL, Lee LH, Agnew MK, Cassels FJ, Scott DA, Whittam TS, Savarino SJ. Evolutionary and functional relationships of colonization factor antigen i and other class 5 adhesive fimbriae of enterotoxigenic *Escherichia coli*. Infect Immun. 2004; 72:7190–7201. [PubMed: 15557644]
 14. Low, D.; Braaten, B.; Van der Woude, M. Fimbriae in *Escherichia coli* and *Salmonella*. In: Neidhardt, FC.; Curtiss, R., III; Ingraham, JL.; Lin, ECC.; Low, KB.; Magasanik, B., et al., editors. Cellular and Molecular Biology. American Society for Microbiology; Washington, D.C: 1996. p. 146-157.
 15. Chattopadhyay S, Tchesnokova V, McVeigh A, Kisiela DI, Dori K, Navarro A, Sokurenko EV, Savarino SJ. Adaptive evolution of class 5 fimbrial genes in enterotoxigenic *Escherichia coli* and its functional consequences. J Biol Chem. 2012; 287:6150–6158. [PubMed: 22215679]
 16. Baker KK, Levine MM, Morison J, Phillips A, Barry EM. CfaE tip mutations in ETEC CFA/I fimbriae define critical human intestinal binding sites. Cell Microbiol. 2009
 17. Madhavan TP, Sakellaris H. Colonization factors of enterotoxigenic *Escherichia coli*. Advances in applied microbiology. 2015; 90:155–197. [PubMed: 25596032]
 18. Tacket CO, Maneval DR, Levine MM. Purification, morphology, and genetics of a new fimbrial putative colonization factor of enterotoxigenic *Escherichia coli* O159:H4. Infect Immun. 1987; 55:1063–1069. [PubMed: 2883122]
 19. Hall RH, Maneval DR Jr, Collins JH, Theibert JL, Levine MM. Purification and analysis of colonization factor antigen I, coli surface antigen 1, and coli surface antigen 3 fimbriae from enterotoxigenic *Escherichia coli*. J Bacteriol. 1989; 171:6372–6374. [PubMed: 2572583]
 20. Evans DG, Evans DJ Jr, Clegg S, Pauley JA. Purification and characterization of the CFA/I antigen of enterotoxigenic *Escherichia coli*. Infect Immun. 1979; 25:738–748. [PubMed: 39896]
 21. Yoshimatsu K, Yuyama Y, Ono E, Syuto B, Naiki M. New methods for isolation of K99 fimbriae from enterotoxigenic *Escherichia coli*. The Journal of veterinary medical science/the Japanese Society of Veterinary Science. 1991; 53:1119–1121. [PubMed: 1686410]
 22. Amorim CR, Matsuura MS, Rosa JC, Greene LJ, Leite DS, Yano T. Purification and characterization of the fimbria F18ac (2134P) isolated from enterotoxigenic *Escherichia coli* (ETEC). Vet Microbiol. 2000; 76:41–49. [PubMed: 10925040]
 23. Barry EM, Altboum Z, Losonsky G, Levine MM. Immune responses elicited against multiple enterotoxigenic *Escherichia coli* fimbriae and mutant LT expressed in attenuated *Shigella* vaccine strains. Vaccine. 2003; 21:333–340. [PubMed: 12531629]
 24. Noriega FR, Losonsky G, Wang JY, Formal SB, Levine MM. Further characterization of delta *aroA* delta *virG* *Shigella flexneri* 2a strain CVD 1203 as a mucosal *Shigella* vaccine and as a live-vector vaccine for delivering antigens of enterotoxigenic *Escherichia coli*. Infect Immun. 1996; 64:23–27. [PubMed: 8557344]
 25. Simon R, Curtis B, Deumic V, Nicki J, Tennant SM, Pasetti MF, Lees A, Wills PW, Chacon M, Levine MM. A scalable method for biochemical purification of *Salmonella* flagellin. Protein Expr Purif. 2014; 102:1–7. [PubMed: 25050462]

26. Tacket CO, Losonsky G, Link H, Hoang Y, Guesry P, Hilpert H, Levine MM. Protection by milk immunoglobulin concentrate against oral challenge with enterotoxigenic *Escherichia coli*. N Engl J Med. 1988; 318:1240–1243. [PubMed: 3283555]
27. Stahlhut SG, Struve C, Krogfelt KA, Reisner A. Biofilm formation of *Klebsiella pneumoniae* on urethral catheters requires either type 1 or type 3 fimbriae. FEMS Immunol Med Microbiol. 2012; 65:350–359. [PubMed: 22448614]
28. Mulvey MA, Lopez-Boado YS, Wilson CL, Roth R, Parks WC, Heuser J, Hultgren SJ. Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. Science. 1998; 282:1494–1497. [PubMed: 9822381]

HIGHLIGHTS

- ETEC fimbriae expressed from plasmids in *E. coli* production strains
- Orthogonal purification steps: size, solubility, charge
- Anion-exchange membranes used to capture fimbriae
- Fimbriae highly purified, recognized by convalescent human sera

Figure 1. Harvest, centrifugation and precipitation of extracted fimbriae

(A.) SDS-PAGE with Coomassie staining for extracted and fractionated CS4 fimbrial proteins. Lane: M. Molecular weight (kDa) marker, 1. growth culture pellet, 2. growth culture supernatant, 3. post-shearing supernatant, 4. $39,000 \times G$ supernatant, 5. $39,000 \times G$ pellet, 6. 15% ammonium sulfate supernatant, 7. 15% ammonium sulfate pellet; (B.) SDS-PAGE (upper panel, Lane M, Molecular weight marker) and densitometry quantitation of the ~12 kDa band (lower panel) in supernatant fractions after precipitation of CFA/I major fimbrial protein CfaB from ultracentrifugation supernatants with various concentrations of ammonium sulfate as indicated.

Figure 2. Anion exchange membrane chromatography on fractionated CS4 fimbriae
A. SDS-PAGE/Coomassie: Lane M. Molecular weight (kDa) marker, 1. dialyzed ammonium sulfate pellet, 2–3. representative flow-through fractions, 4–7. 100 mM NaCl eluate fractions, 8–9. 1M NaCl eluate fractions; (B.) Chromatogram measuring absorbance at 280 nm (solid, black line) and 252 nm (dashed grey line).

Figure 3. SDS-PAGE analysis of final purified fimbriae

Coomassie stain for (A.) Lane M. Molecular weight (kDa) marker, 1. 20 μ g CS4 and (B.) Lane M. Molecular weight (kDa) marker, 1. 8 μ g CFA/I; Western blot for (C.) 2 μ g purified CS4 with polyclonal anti-CS4 guinea pig sera and (D) 0.4 μ g purified CFA/I with polyclonal anti-CFA/I guinea pig sera.

Figure 4. Structural analysis of purified fimbriae

(A.) Purified CFA/I fimbriae were assessed by HPLC-SEC through a Biosep SEC4000 column at 1 ml/minute in PBS with monitoring of UV absorbance at 280 nm (thick dark line) and 252 nm (light dashed line). Total excluded material elutes at ~ 5.5 minutes based on gel filtration standards; Electron microscopy of purified (B) CFA/I and (C) CS4, 100 nm scale indicated by solid bar.

Figure 5. Recognition of purified CFA/I fimbriae protein preparations by convalescent human sera

Fimbriae were coated onto microtiter plate wells at 5 µg/ml or 10 µg/ml and assessed by ELISA with different dilutions of anti-sera from volunteers infected with ETEC. Results represent average absorbance values from replicate wells.