TYPE 1 FIMBRIAL ADHESIN IN HOST INTERACTION

THE TYPE 1 FIMBRIAL ADHESIN MEDIATES THE INTERACTION OF ADHERENT-INVASIVE *ESCHERICHIA COLI* WITH THE HOST

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ABSTRACT

Crohn's Disease is a chronic inflammatory bowel disease characterized by an overzealous immune response to a microbial trigger in genetically susceptible individuals. Although this microbial trigger is unknown, *Escherichia coli* with adherent and invasive properties (Adherent-Invasive *Escherichia coli*, AIEC) is preferentially enriched in a proportion of Crohn's Disease patients. AIEC can adhere to and invade intestinal epithelial cells and replicate intracellularly within epithelial cells and macrophages *in vitro*. One important colonization factor expressed by AIEC is the type 1 fimbrial adhesin protein FimH. FimH mediates colonization of CEABAC10 transgenic mice and can bind several host cell receptors including the macrophage receptor CD48 *in vitro* indicating a potential role for FimH in macrophage interaction. However, it was not known whether FimH contributed to phagocytosis of AIEC or colonization of wild-type mice. Here we show that FimH enhances early intracellular AIEC levels *in vitro* and colonization *in vivo*. We found that deletion of *fimH* may reduce intracellular AIEC burden at 2 hours post-infection and that this effect was modulated by bacteria opsonisation. Using a competitive index assay, we show that a Δ*fimH* mutant is unable to chronically colonize CD-1 mice at the same levels as the parental strain. Our results demonstrate that FimH is an important AIEC colonization factor and may increase interaction with macrophages. Identifying factors such as FimH which contribute to colonization and persistence will further our understanding of AIEC survival strategies within the host. Development of therapeutics targeting FimH may provide a means to reduce harmful bacteria overgrowth particularly after surgical intervention.

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List of all Abbreviations and Symbols

AIEC – Adherent-Invasive *Escherichia coli*
AP-2 – Adaptor protein complex-2
APEC – Avian pathogenic *Escherichia coli*
ATG16L1 – Autophagy-related protein 16-1
BSA – Bovine serum albumin
CARD15 – Caspase recruitment domain-containing protein-15
CD – Crohn's Disease
CEACAM6 – Carcinoembryonic antigen-related cell adhesion molecule 6
CFU, CFU/g or CFU/mL – Colony forming unit, Colony forming unit per gram or Colony forming unit per millilitre
CI – Competitive index
DMEM – Dulbecco's Modified Eagle Medium
E. coli – *Escherichia coli*
FAK – Focal adhesion kinase
FACS – Fluorescence activated cell sorting
FBS – Fetal bovine serum
FITC – Fluorescein isothiocyanate
FT - Flowthrough
GP2 – Glycoprotein 2
Gp96 – Glycoprotein 96
GPI – Glycosylphosphatidylinositol
HMDM – Human monocyte-derived macrophage
HRP – Horseradish peroxidase
IBD – Inflammatory Bowel Disease
IEC – Intestinal epithelial cell
IL – Interleukin
IRF3 – Interferon regulatory factor 3
IRGM – Immunity-related GTPase family, M
IFN – Interferon
IPTG – Isopropyl β-D-1-thiogalactopyranoside
Lamp-1 – Lysosomal-associated membrane protein-1
LB – Luria Bertani
LC3 – Light chain 3
M cell – Microfold cell
MOI – Multiplicity of infection
MyD88 – Myeloid differentiation primary response gene 88
NOD2 – Nucleotide binding oligomerization domain-containing 2
OD_{600} – Optical density at 600 nanometres
OmpA – Outer membrane protein A
OmpC – Outer membrane protein C
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction

PE – Phycoerythrin
PI-3K – Phosphoinositide-3 kinase
RPM – Rotations per minute
SDS – Sodium dodecyl sulfate
T1F – Type 1 fimbriae
TBST – Tris-buffered saline, 0.1% Tween-20
TLR – Toll-like receptor
TNF – Tumor necrosis factor
Tram – TRIF-related adaptor molecule
Trif – TIR domain-containing adapter molecule 1
UPEC – Uropathogenic *Escherichia coli*
α – Alpha
β – Beta
γ – Gamma
Δ – Delta
λ – Lambda

Declaration of Academic Achievement

LEW and BKC conceived and designed experiments; LEW performed experiments and analyzed data; Sarah Reid-Yu performed flow cytometry, analyzed flow cytometric data and sacrificed mice; Dr. Joseph McPhee inoculated mice with bacteria; Dr. Cherrie Small inoculated mice with bacteria and sacrificed mice; Kun Zhang developed protein purification protocol and performed protein purification.

Introduction

Crohn's disease

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) that can affect any part of the gastrointestinal tract but commonly manifests in the ileum and colon (5). CD is characterized by discontinuous, transmural inflammation which appears as a cobblestone pattern along the intestinal mucosa (98). Chronic unresolved inflammation can promote bowel constriction, transmural ulceration, development of abscesses and fistulae and formation of non-caseating granulomas (117). CD can also manifest as extra-intestinal inflammation within the joints, liver, spine and skin (12).

Epidemiological studies indicate that CD is more prevalent in developed countries such as Canada and Northern Europe (15, 87). However, prevalence is increasing in developing nations concomitant with industrialization (111, 161). Recent studies estimate that 81 000 Canadians and 436 000 Americans are living with CD (15, 71). Increasing global prevalence and lack of a cure supports the need to understand the etiopathogenesis of CD. Although this is not yet well understood, it is increasingly clear that CD is a multi-factorial disease requiring a confluence of factors in order to occur. These factors can be divided into four categories: environment, genetics, immune response and gut microbiota (128).

Environment

Monozygotic twin studies have shown that IBD does not necessarily affect both twins despite an identical genetic background suggesting that disease development is

influenced by the environment (55, 146, 152). First-generation children of parents who have immigrated to an area with increased IBD incidence have a greater risk of developing IBD suggesting exposure to novel environmental factors has increased disease risk (115). Environmental factors that may increase disease risk include smoking, antibiotic use, dietary changes and decreased exposure to infectious agents (14). Although the mechanisms by which these factors promote IBD is unclear, it is known that many of these factors can affect the gut microbiota and mucosal immunity (14).

Genetics

Genome-wide association studies have uncovered CD-associated genetic variants in microbial sensing, autophagy and immunity (31, 128). For instance, single nucleotide polymorphisms (SNPs) in the innate pattern recognition receptor *NOD2*/*CARD15* have been identified (61, 109). Nucleotide binding oligomerization domain-containing 2 (NOD2) recognizes muramyl dipeptide, a component of peptidoglycan in the bacterial cell wall (52, 64). NOD2 is constitutively expressed by dendritic cells, monocytes, macrophages and Paneth cells which secrete antimicrobial peptides (16). CD-associated polymorphisms in *NOD2* are loss-of-function mutations (2) which may alter sensing of peptidoglycan, secretion of anti-microbial peptides and immune tolerance in the gut (25, 153, 157). SNPs have also been identified in the autophagy genes *ATG16L1* (56) and *IRGM* (94). The ATG16L1 T300A CD variant impaired autophagic processing of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and Adherent-Invasive *Escherichia coli* (AIEC) strain LF82 (80, 82). Knockdown of IRGM enhanced

intracellular replication of LF82 in epithelial cells (82). Together, these polymorphisms along with others suggest an altered microbial processing in CD.

Immunity

The immense bacterial burden within the gut lumen is separated from resident lamina propria immune cells by a single epithelial cell layer. In CD, a chronic aberrant immune response to the gut microbiota is facilitated by alterations in antimicrobial secretion, handling of intracellular bacteria and loss of epithelial integrity (82, 157, 160). This results in enhanced bacterial translocation and persistence, chronic activation of resident and recruited immune cells and increased pro-inflammatory cytokine production culminating in epithelial injury and ulceration (53, 67, 97, 132, 160). The lamina propria contains the largest resident macrophage population. Under normal physiological conditions, these resident macrophages help to maintain an immunosuppressive environment by downregulating expression of certain immune receptors and pro-inflammatory cytokines (140, 141, 142). IBD patients have increased numbers of activated macrophages which home to the inflamed gut mucosa (54, 70). These macrophages exhibit defective vesicular trafficking which was shown to alter cytokine secretion in response to *Escherichia coli* (*E. coli*) (138).

Gut microbiota

Both clinical and experimental evidence implicate the gut microbiota in initiation of inflammation. Clinically, diversion of the fecal stream prevents inflammation

following surgical resection in CD patients and antibiotics have successfully been used to treat a subset of CD patients (47, 123). In several mouse models, gut microbiota are required to induce spontaneous colitis (134, 155). In a recent study, Bacteroides singularly induced colitis in antibiotic-treated colitis-susceptible mice (17). Distinct changes in the gut microbiota of CD patients characterized by an increase in γ-Proteobacteria and a decrease in Firmicutes has been shown although the consequence of this is not well understood (13, 49, 84, 90). Repeatedly, *E. coli* has been detected at abnormally predominant levels in the gut microbiota of CD patients suggesting that *E. coli* may be involved in CD pathogenesis (13, 49, 77, 84, 107).

Escherichia coli in Crohn's Disease

E. coli is a commensal organism that exists within the gut lumen or mucus layer that covers the intestinal epithelium (88). *E. coli* was first implicated in CD when it was shown that CD patients had an increased antibody titer to *E. coli* relative to healthy controls suggesting an abnormal anti-*E. coli* immune response (150). This was later verified by several independent studies which found that an increased proportion of CD patients displayed elevated levels of antibodies directed against the outer membrane protein C (OmpC) relative to ulcerative colitis patients and healthy controls in which antibody levels correlated with the need for intestinal surgery (6, 100, 101). Using polyclonal *E. coli* antibodies, CD biopsy specimens were positively labeled particularly within lamina propria macrophages, granulomas and near fissures and ulcerations (26, 85). Refined analysis of microdissected granulomas detected *E. coli* DNA in 80% of CD

patients but only 10% of control patients (124). Numerous studies have reported increased numbers of mucosa-associated *E. coli* in CD although the consequence of this has remained speculative (13, 36, 39, 77, 91, 107, 149). For instance, Neut *et al*. found that CD patients with early post-surgical endoscopic recurrence had higher counts of *E. coli* than those with recurrence after one year suggesting a role in early lesion development (107). Another study similarly found that *E. coli* was preferentially isolated from early recurrent ileal lesions in CD patients than control patients (38). Using 16S rDNA libraries, Baumgart *et al*. found that *E. coli* is enriched in CD ileitis and *E. coli* levels correlated with disease severity (13). Further characterization of these isolates found an increased prevalence of the B2 phylogroup associated with extraintestinal pathogenic *E. coli* and *in vitro* adhesive and invasive properties (13, 38, 39, 77, 91, 135). One study found that ~80% of *E. coli* isolated from CD lesions were adherent to Caco-2 intestinal epithelial cells versus 33% of controls (39). Similarly, another study found that 86% of IBD isolates were adherent to buccal epithelial cells versus 27% from infectious diarrhea and none from controls (24). An increased prevalence of invasive *E. coli* isolates has also been shown in several studies (38, 91). Together, these data provide support for the role of *E. coli* in CD pathogenesis.

The discovery that CD *E. coli* isolates have both *in vitro* adhesive and invasive properties led to a newly designated pathovar: Adherent-Invasive *Escherichia coli* (AIEC) (20). AIEC is defined by its ability to adhere to and invade intestinal epithelial cells (20, 38, 39), induce rearrangement of the host cytoskeleton (20), form a replicative niche within macrophages without inducing apoptosis (23, 53) and induce secretion of

tumor necrosis factor alpha (TNF-α) by infected host cells (53). Currently, two prototypic AIEC strains LF82 and NRG857c independently isolated from CD ileal tissue biopsies are being characterized within our lab with LF82 being the predominantly studied strain within the scientific field (96, 105). Four AIEC genome sequences including LF82 and NRG857c have been published (35, 78, 96, 105). Interestingly, these genomes lack many common virulence determinants associated with pathogenic *E. coli* or other enteric pathogens.

Adherence and Invasion of Epithelial Cells

LF82 can adhere to and invade several different epithelial cell lines including HEp-2, Intestine-407, Caco-2 and HCT-8 (20). Invasion is dependent on actin microfilament and microtubule rearrangement which induces membrane extensions around adherent bacteria (20). Invasion increases cellular permeability which was associated with rearrangement of the tight junction protein zonula occludens-1 (106, 158). Once invaded, LF82 is targeted to the autophagy pathway and can replicate intracellularly within autophagosomes (20, 82, 158). LF82 expresses several virulence factors associated with adherence and invasion. Using a Tn*phoA* insertion mutagenesis screen, Boudeau *et al*. found that the majority of non-invasive mutants had insertions within the type 1 fimbriae (T1F) operon. Later studies demonstrated that adherence of LF82 to primary CD ileal enterocytes required the interaction of type 1 pili with the host receptor carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) (11). Adherence and invasion are further mediated by several other factors. Flagella are required for

motility and were found to regulate T1F expression (10). A nonflagellated *fliC* mutant had significantly decreased adherence and invasion despite normal T1F expression suggesting that flagella can independently mediate adherence and invasion (45). Flagella and T1F expression are co-regulated by several proteins including Fis histone-like protein and the RNA chaperone Hfq (95, 136). T1F expression was also found to be regulated by the periplasmic oxidoreductase DsbA (21). NlpI and YfgL are lipoproteins implicated in adherence and invasion (9, 121). YfgL was found to affect outer membrane vesicle formation which may mediate invasion (121). The outer membrane proteins OmpC as a regulatory protein and OmpA as a ligand for the host receptor Gp96 are also required for adherence and invasion (120, 122). LF82 can interact with specialized epithelial cells termed microfold cells (M cells) located in Peyer's patches within the gut via long polar fimbriae (29). Thus, LF82 expresses several factors to promote adhesion and invasion of epithelial cells *in vitro*.

Survival and replication within macrophages

LF82 has been shown to survive and replicate within large phagosomes without inducing necrosis or apoptosis (22, 53). In a seminal study, 15 AIEC strains were shown to replicate extensively within macrophages with replication rates ranging from 2.51- to 35.4-fold at 26 hours post-infection in J774-A1 macrophage-like cells (53). The replication rate of LF82 in these cells was ~5-fold at 26 hours post-infection. LF82 was also able to replicate in human monocyte-derived macrophages (HMDMs) with a replication rate of >2-fold but was unable to survive in murine peritoneal macrophages

where only 14.2% of the initial intracellular burden remained at 26 hours post-infection (53). Transmission electron microscopy of LF82-infected J774-A1 cells showed large phagosomes containing numerous LF82 bacteria (53). These phagosomes were shown to successively acquire the early endosomal marker early endosome antigen-1 and late endosomal markers Rab7 and lysosomal-associated membrane protein-1 (Lamp-1) (23). These phagosomes matured into Lamp-1+, Lamp-2+, cathepsin D+ phagolysosomes where acidic pH promoted intracellular replication (23). More recently, phagocytosis of LF82 by THP-1 macrophage cells was shown to activate the autophagy pathway (81). In this study, intracellular survival was dependent on host cell targeting. LF82 targeted along the autophagy pathway within light chain 3 (LC3)-positive phagosomes was unable to survive whereas LF82 within LC3-negative phagosomes exhibited a <80% survival rate at 24 hours post-infection (81). Functional inhibition of the autophagy pathway by siRNA knockdown of two autophagy proteins ATG16L1 or IRGM increased intracellular survival. Similarly, intracellular survival was increased in *Nod2*-knockout macrophages (81). This data is particularly relevant to CD where single nucleotide polymorphisms in *Atg16L1*, *IRGM* and *Nod2* have been identified (2, 56, 94).

Other CD *E. coli* isolates have similarly been shown to survive and replicate within macrophages. 7 CD colonic isolates and 6 control isolates were able to replicate with a mean rate of 6.36-fold and 5.2-fold respectively in J774-A1 cells and the CD isolate HM605 replicated 3.9-fold in HMDMs at 8 hours post-infection (148). The CD isolate EC10 and LF82 were shown to survive in RAW264.7 cells with <2-fold replication rate at 26 hours post-infection (106). Similarly, a collection of CD isolates

were able to survive in RAW264.7 cells with a mean survival rate of 60.02% at 20 hours post-infection versus 51.36% for control isolates (135).

LF82-infected J774-A1 cells were shown to secrete the pro-inflammatory cytokine TNF-α (53). More recent work has demonstrated that TNF-α secretion correlates with intracellular replication (22). Bringer *et al*. demonstrated a positive correlation between TNF-α levels and multiplicity of infection (MOI). Inhibiting intracellular replication using a replication-defective mutant or via chemical inhibition decreased TNF-α secretion. Conversely, TNF-α secretion was unaffected by the initial intracellular burden. Exogenous TNF-α treatment enhanced intracellular replication in a dose-dependent manner suggesting a positive feed-back loop between TNF-α secretion and intracellular replication (22).

Together, the ability to adhere to and invade epithelial cells and survive within macrophages may facilitate chronic AIEC colonization.

Type 1 fimbriae: Structure and assembly

One of the most well-studied examples of adhesive surface organelles expressed by enteric and urinary pathogens such as *E. coli*, *Salmonella enterica* and *Klebsiella pneumoniae* is type 1 fimbriae (T1F) (42, 43, 44). T1F are inflexible thin rod-like filaments that can extend up to 3 microns from the bacterial surface and are distinguished by their ability to agglutinate guinea pig erythrocytes or yeast cells in a mannose-sensitive manner (108, 125, 131). T1F are encoded by the *fim* operon which undergoes phase variable expression (3, 112, 131). Phase variation is mediated by FimB and FimE

recombinases which catalyze the inversion of specific sequences within the *fimA* promoter to either activate or repress expression of downstream genes (3, 50). FimA is the major fimbrial subunit with ~1000 copies per fimbriae (74, 130). FimI is a protein of unknown function. FimC and FimD form a chaperone-usher pair which assembles T1F via the chaperone-usher pathway (76). FimC is the periplasmic chaperone which binds fimbrial subunits via donor strand complementation in which the N-terminal G1 strand of FimC interacts with a hydrophobic groove on the subunit surface to catalyze folding, prevent aggregation and target the chaperone-subunit complex to the outer membrane usher, FimD (32, 68, 75, 131). Binding of the chaperone-subunit complex to FimD induces a conformational change that opens the translocation channel (114). FimD facilitates subunit-subunit interaction via donor strand exchange in which the N-terminal strand of the incoming subunit binds the same hydrophobic groove of the bound chaperone-subunit complex, dissociating FimC (114). The affinity of different chaperone-subunit complexes for FimD determines the order in which subunits are added (129). FimF and FimG are adaptor proteins which connect the rigid fimbrial stalk to the more flexible fimbrial tip and FimH is the mannose-binding adhesin (69, 79, 131).

FimH has an N-terminal lectin domain which contains the highly conserved mannose binding pocket and a C-terminal pilin domain (32). FimH can bind receptors on several epithelial cell types including intestinal and bladder epithelium (11, 46) and cells involved in innate immunity including mast cells (89) and macrophages (8) (Figure 1). Binding specificity is partially determined by single nucleotide polymorphisms within *fimH* (144). For instance, monomannose binding affinity is higher in FimH expressed by

urinary tract versus fecal *E. coli* isolates which has been attributed to several missense mutations (143). The presence of mono-mannosylated receptors in the urinary tract likely selects for FimH variants that bind monomannose and may contribute to the tissue tropism of *E. coli* (145). The ability of FimH to bind receptors on the surface of epithelial and immune cells suggests that T1F are important for colonization and activation of the innate immune response.

Breaching the first line of defense

Microfold cells

Microfold cells (M cells) are rare, interspersed epithelial cells within follicle-associated epithelium that are specialized to transcytose lumenal intestinal antigen and bacteria to the underlying lymphoid follicles which contain antigen-presenting cells that can initiate an immune response (126, 151). Because of this specialized function, M cells are targeted by the enteric pathogens *S.* Typhimurium, *Shigella flexneri* and *Yersinia pseudotuberculosis* to gain access to the lamina propria (33, 34, 156). M cells express the glycosylphosphatidylinositol-anchored (GPI-anchored) protein, glycoprotein 2 (GP2) on the apical surface and on endosome-like organelles (59). Recombinant GP2 was shown to bind FimH-expressing bacteria *in vitro* but not a Δ*fimH* mutant or bacteria which do not express T1F (59). Binding of GP2 via FimH was crucial for Salmonella pathogenesis *in vivo*. Salmonella invasion of Peyer's patches and translocation to mesenteric lymph nodes and generation of an antibody-mediated response was drastically reduced in $Gp2^{-/-}$ mice or in a Δ*fimH* mutant (59). In contrast, invasion of *Yersinia enterocolitica*, a bacterium

that does not express FimH, was unaffected in *Gp2*$^{-/-}$ mice (59). This elegant study identified GP2 as the FimH receptor on M cells and demonstrated the importance of this interaction for enteric pathogenesis *in vivo*.

Intestinal epithelial cells

Intestinal epithelial cells (IECs) constitute a single layer of cells that maintain gut homeostasis by providing a barrier between gut commensal bacteria and lamina propria immune cells. When this epithelial barrier is breached by intestinal bacteria, these immune cells become activated and initiate a pro-inflammatory response (139). In CD, this response is chronic and can lead to tissue injury, scarring and fibrosis (127).

AIEC adherence and invasion of IECs was shown to require T1F *in vitro* (19). However, expression of AIEC *fim* genes in a non-pathogenic *E. coli* strain did not confer invasiveness suggesting that other invasive factors are required (19). Adherence of AIEC to isolated ileal enterocytes from CD patients required FimH (11). GPI-anchored CEACAM6 was identified as the FimH receptor on ileal enterocytes (11). CEACAM6 expression is up-regulated in the ileum of CD patients and in response to the pro-inflammatory cytokines interferon gamma (IFN-γ) and TNF-α and LF82 invasion (11, 106). T1F were required for invasion of CEACAM6-expressing colonic tissue *ex vivo* and colonization of CEABAC10 mice which express human CEACAM6 (27, 41). Downstream signaling events have not yet been elucidated. Thus, FimH-mediated interaction with CEACAM6 promotes adherence to IECs and colonization of the murine intestine.

Interacting with the innate immune system

Macrophages

Macrophages are mononuclear, phagocytic cells located in subepithelial regions which participate in immune homeostasis and in mounting an innate immune response to foreign antigen. During bacterial infection, macrophages ingest and kill bacteria that have breached the epithelial barrier and release an array of pro-inflammatory cytokines and chemokines to induce a localized immune response (139). Macrophages express a plethora of pattern recognition receptors which sense conserved microbial motifs to initiate phagocytosis (139). FimH-expressing bacteria were found to be preferentially bound by peritoneal macrophages, bone marrow-derived macrophages and macrophage-like cells *in vitro* suggesting that FimH contributes to macrophage recognition and uptake (57). FimH-expressing bacteria are internalized via a pathway that is distinct from antibody-mediated internalization characterized by small, less acidified phagosomes and an attenuated oxidative burst (8). This decrease in bactericidal activity correlated with increased intracellular survival although other studies have not found that FimH contributes to intracellular survival (8, 22, 57). The GPI-anchored receptor CD48 was identified as the FimH receptor (8). Anti-CD48 antibody prevented phagocytosis of FimH-expressing bacteria in a dose-dependent manner whereas a control antibody had no effect. Disruption of lipid raft microdomains, often enriched in GPI-anchored proteins, inhibited internalization of FimH-expressing bacteria in a dose-dependent manner but had no effect on antibody-mediated internalization (8). Thus, FimH interaction with CD48

activates a distinct mechanism of macrophage phagocytosis that may contribute to intracellular survival.

Host response

FimH-mediated interaction with the host induces an inflammatory immune response. Purified FimH induced MyD88-dependent TNF-α and nitric oxide production by RAW264.7 macrophage-like cells *in vitro* (99), and TNF-α and interleukin-12 (IL-12) production by alveolar macrophages *ex vivo* (1). In order to elicit this response, FimH directly binds and activates Toll-like receptor 4 (TLR-4) in the absence of lipopolysaccharide signaling (99). This response was protective against viral challenge *in vitro* and *in vivo*, and was shown to require MyD88, Trif, IFN-α/βr and IRF-3 *in vitro* and MyD88, IFN-β, and TLR-4 *in vivo* (7). During uropathogenic *E. coli* (UPEC) infection, FimH enhanced bladder colonization and neutrophil recruitment as a Δ*fimH* mutant had reduced colonization and neutrophil recruitment relative to wild-type UPEC. This neutrophil response was dependent on TLR-4 as no neutrophil recruitment in response to UPEC was observed in TLR-4-deficient mice (7). These mice also had higher bacterial burden in their bladders suggesting that TLR-4 signaling contributes to clearance. During enteric AIEC infection, FimH was required for development of colitis and induction of a pro-inflammatory immune response measured by IL-1β, IL-6 and IL-17 mRNA levels (27). Thus, FimH is able to signal via TLR-4 and activate an innate immune response both *in vitro* and *in vivo*.

Hypothesis

Since macrophages are important in the inflammatory cascade in CD and in AIEC pathogenesis and since FimH can promote bacteria binding to macrophages, then we suggest that **FimH contributes to the interaction of AIEC with macrophages**.

Results

Type 1 fimbriae are expressed under static conditions.

Previous studies have shown that *E. coli* expresses T1F after static growth (30, 62). To verify that NRG857c, LF82 and K-12 express T1F under the static growth condition used, a yeast agglutination assay was performed in the absence and presence of a 2.5% D-mannose solution (Figure 2). In Figure 2, all wild-type strains were able to agglutinate yeast in a mannose-sensitive manner after static growth indicating positive T1F expression. T1F expression was unaffected by the complementation vector pBADGr (Figure 2A) or by the deletion of *phoP* (Figure 2B). As a negative control, isogenic Δ*fimH* mutant strains were unable to agglutinate yeast indicating that FimH is required for functional T1F expression. This phenotype could be partially restored by expression of *fimH* from pBADGr (Figure 2A). Thus, T1F are expressed under static growth conditions.

The FimH receptor CD48 is expressed by RAW264.7 macrophage-like cells.

FimH was previously shown to bind CD48 on the surface of murine bone marrow derived macrophages and mast cells (8, 89). To determine if RAW264.7 cells

express CD48, RAW264.7 cells were stained with a phycoerythrin (PE) hamster anti-mouse CD48 antibody and analyzed by flow cytometry. In Figure 3, 29.8% of the total population were positive for surface CD48 expression (Figure 3B). Unstained cells and cells stained with an isotype control antibody served as negative controls (Figure 3A&C). Splenocytes which have high CD48 expression were used as a positive control (data not shown). Thus, RAW264.7 cells can express the FimH receptor CD48.

Loss of FimH may reduce intracellular bacteria levels at 2 hours post-infection under non-opsonic conditions *in vitro*.

FimH was previously shown to enhance macrophage binding of *E. coli* (18, 57). Given that macrophages have been implicated in AIEC pathogenesis, we wanted to determine if FimH contributes to early intracellular AIEC levels. Using a gentamicin protection assay, RAW264.7 cells or J774-A1 cells were infected with either wild-type or Δ*fimH* mutant strains (Figure 4). At 2 hours post-infection, a trend towards lower NRG857c Δ*fimH* intracellular bacteria levels relative to NRG857c was observed under all experimental conditions tested although this difference was not statistically significant (Figure 4). In contrast, a trend towards lower LF82 Δ*fimH* intracellular levels was only observed at a low MOI in RAW264.7 cells (Figure 4A) but not at a high MOI in RAW264.7 cells or J774-A1 cells (Figure 4B&C). A trend towards lower K-12 Δ*fimH* intracellular bacteria levels was also observed indicating that this effect may be common to both the AIEC strain NRG857c and non-pathogenic K-12 under all conditions tested. Together these data suggest that FimH may increase early intracellular levels of

NRG857c and K-12 and has a limited effect on intracellular LF82 levels under non-opsonic conditions.

Loss of FimH does not reduce early intracellular AIEC levels after bacteria opsonisation.

To more closely mimic the opsonin-rich environment of the gut lumen, bacteria were opsonised with normal human serum prior to infection. At 2 hours post-infection, there was no statistically significant difference between wild-type and Δ*fimH* intracellular bacteria levels (Figure 5). Opsonisation appeared to increase the levels of intracellular NRG857c and NRG857c Δ*fimH* in RAW264.7 cells and J774-A1 cells where the levels of NRG857c Δ*fimH* surpassed that of the wild-type (Figure 5). This suggests that opsonisation of NRG857c Δ*fimH* compensates for the loss of FimH. Similarly, opsonisation also increased the intracellular levels of LF82 Δ*fimH* and K-12 Δ*fimH* to levels at or above wild-type levels in J774-A1 cells (Figure 5B). Oddly, opsonisation did not affect the levels of LF82 or K-12 in RAW264.7 cells. This may reflect lower opsonisation of these strains during incubation with human serum. These data suggest that loss of FimH does not affect early intracellular levels under opsonic conditions.

FimH may contribute to intracellular survival.

One of the defining characteristics of AIEC is the ability to survive and replicate within macrophages (53). Using a standard gentamicin protection assay, we were unable to demonstrate extensive replication at 2 and 20 hours post-infection despite varying the

cell line, MOI and bacteria opsonisation. A >1-fold increase was observed in RAW264.7 cells infected with opsonised NRG857c and K-12 at a high MOI (Figure 6B). Under these conditions, it appears that FimH may contribute to intracellular survival although statistical significance was not achieved. There was a statistically significant difference between the fold survival of NRG857c and LF82 under these conditions. However, neither survival nor replication was seen under the majority of conditions tested at 2 and 20 hours post-infection. NRG857c Δ*phoP* served as an attenuated control. In the absence of intracellular survival, the effect of FimH on intracellular bacteria levels was variable. At a high MOI, there was a trend towards enhanced intracellular bacteria levels of LF82 upon loss of FimH (Figure 6B-D). In contrast, a trend towards decreased intracellular bacteria levels of K-12 was observed upon loss of FimH. Thus, it appears that the effect of FimH on intracellular survival is variable depending on the strain background and experimental condition.

FimH does not affect TNF-α secretion.

FimH has been shown to induce TNF-α secretion by macrophages *in vitro* and TNF-α is likely a primary inflammatory mediator in CD (7, 22, 99, 128, 147). To determine if FimH affects TNF-α secretion, TNF-α levels were measured in cell supernatants collected at 2h (Figure 7A&B) or 20h post-infection (Figure 7C) from wild-type or Δ*fimH*-infected cells. Loss of FimH did not affect TNF-α levels at either 2 hours or 20 hours post-infection as no statistically significant difference between TNF-α levels

induced by wild-type or Δ*fimH* strains was observed. It is likely that TNF-α secretion is being stimulated by other bacterial antigens in the absence of FimH.

FimH contributes to persistence of AIEC *in vivo*.

Previously, T1F and host CEACAM6 were shown to be important for LF82 colonization of CEABAC10 transgenic mice (28). However, both NRG857c and LF82 have been found to colonize wild-type mice in the absence of CEACAM6 suggesting that CEACAM6 is not necessary for colonization (Submitted June 11 2012 to PLoS Pathogens). To determine whether FimH contributes to AIEC colonization in wild-type mice, competitive index (CI) experiments in which NRG857c was competed against NRG857c Δ*fimH* and LF82 was competed against LF82 Δ*fimH* in CD-1 mice were performed (Figure 8&9). By day 7 post-infection, the mean CI value was significantly less than one for NRG857c competed against NRG857c Δ*fimH* indicating that Δ*fimH* has a competitive disadvantage *in vivo* (Figure 8A). This disadvantage was maintained up to day 21 which was reflected in a lower Δ*fimH* bacteria burden in the feces (Figure 8E). At day 27, cecal and colonic burden was assessed. The strong competitive disadvantage observed for NRG857c Δ*fimH* was similarly seen in the cecum and colon with a mean CI value significantly less than one (Figure 8B) and lower bacteria burden (Figure 8F). On day 5 and 9 post-infection and in the colon on day 27 post-infection, the mean CI value was significantly less than one for LF82 competed against LF82 Δ*fimH* (Figure 9A&B) which correlated with a lower Δ*fimH* bacteria burden (Figure 9E&F). The low LF82 organ burden and experimental n value may have increased experimental noise which

could account for the observed variability in the fecal and organ bacteria output and lack of statistical significance at all time points tested (Figure 9A,B&D). Together these data show that FimH contributes to chronic AIEC colonization in CD-1 mice.

Discussion

In this study, we present evidence for the role of FimH in macrophage interaction *in vitro* and long-term colonization *in vivo*. Previously, Boner *et al*. demonstrated increased binding of a nonopsonised fimbriated *E. coli* urinary isolate by human peritoneal macrophages relative to *E. coli* lacking fimbriae expression (18). Hamrick *et al*. observed that murine bone marrow-derived macrophages, murine peritoneal macrophages and the cell line macrophages J774 and IC-21 bound higher levels of fimbriated *E. coli* than nonfimbriated *E. coli* regardless of the host background with cell line macrophages exhibiting the highest fimbriated/nonfimbriated ratio bound (57). Similarly, we found that deletion of *fimH* in NRG857c and K-12 trended towards reduced levels of intracellular bacteria in RAW264.7 cells and J774-A1 cells at 2 hours post-infection under nonopsonic conditions. This effect was only observed in LF82 at a low MOI and may reflect differences in strain background. The LF82 FimH variant contains a unique T158P substitution located in the loop region between the pilin and lectin domain (96). Although the effect of this substitution is unknown, other mutations located outside of the mannose binding pocket have altered binding affinity which may affect FimH-mediated host cell interaction (130). We also assessed the role of FimH in macrophage interaction with opsonised *E. coli*. Here we showed that opsonisation may have

compensated for the loss of FimH such that there was no statistically significant difference between the intracellular levels of wild-type and AIEC *fimH* mutants at 2 hours post-infection. Baorto *et al*. demonstrated differences in antibody-mediated versus FimH-mediated phagocytosis of *E. coli* by murine bone-marrow derived macrophages (8). FimH-mediated phagocytosis required the macrophage receptor CD48 and the presence of lipid microdomains. In contrast, antibody-mediated phagocytosis was CD48-independent and was unaffected by disruption of lipid microdomains (8). Thus, bacteria opsonisation can alter macrophage interaction. Here we showed that loss of FimH may only affect macrophage interaction under nonopsonic conditions.

In our results, we observed a trend towards decreased intracellular survival of NRG857c and K-12 Δ*fimH* mutants in RAW264.7 cells and J774-A1 cells infected at an MOI of 100 under opsonic and nonopsonic conditions respectively. However, this effect was not observed under all experimental conditions. In particular, J774-A1 cells infected with opsonised Δ*fimH* bacteria showed no defect in intracellular survival. A defect in intracellular survival was never observed for LF82 Δ*fimH*. This was similarly seen by Bringer *et al*. who reported no difference in intracellular survival between LF82 and a nonfimbriated Δ*fimA* mutant (22). Thus, the role of FimH in the interaction of LF82 with macrophages may be more subtle than in the interaction of NRG857c or K-12 with macrophages. Together, these data suggest that under certain conditions, FimH may modulate intracellular survival of *E. coli*.

While determining the role of FimH in intracellular survival, we noted that AIEC was unable to extensively replicate intracellularly at 2 and 20 hours post-infection under

the experimental conditions tested. The fold survival rate ranged from 0.31-1.30 for NRG857c, 0.06-0.38 for LF82 and 0.20-1.21 for K-12. Under the same experimental conditions, J774-A1 cells appeared to be more bactericidal than RAW264.7 cells. Increased intracellular bacteria levels at 2 hours post-infection did not necessarily correlate with increased survival. For example, opsonisation of NRG857c appeared to increase intracellular bacteria levels at 2 hours in J774-A1 cells but did not increase fold survival relative to nonopsonic conditions. In contrast, increasing the initial intracellular bacteria burden in RAW264.7 cells by increasing the MOI 10-fold did enhance the fold survival of all strains tested. However, despite varying the MOI and cell line used, we were unable to demonstrate extensive intracellular replication as reported in the literature.

A review of the literature reveals a discrepancy in AIEC replication rates. Several studies report extensive AIEC replication ranging from 2.51-35.4-fold replication whereas other studies report <2-fold replication by 18-24 hours post-gentamicin treatment (13, 22, 23, 53, 81, 105, 106, 135, 137, 148). In several studies that report >2-fold replication by 24 hours post-gentamicin treatment, J774 cells are consistently used (13, 21, 22, 53, 148). In those studies where replication is <2-fold, murine peritoneal macrophages, HMDMs, bone-marrow derived macrophages and RAW264.7 cells were used suggesting that cell type may account for some discrepancy (106, 135, 137). However, we do not observe replication in J774-A1 cells which is in accordance with a previous report of >20% survival of NRG857c at 8 hours in J774.1 cells (105). This may be due to differences in passage number, cell culture practices or other experimental conditions. A closer look at the experimental methodology used in previous studies

reveals key differences. In three studies that reported replication in J774 cells, an infection period of 2 hours prior to the addition of gentamicin was used versus 30 minutes in our experiments. This longer infection period may have increased the initial phagocytic load allowing more extensive replication to occur (13, 53, 148). Replication was also observed in two other studies which had a shorter infection period of 20 minutes (22, 23). In these studies, a higher MOI was used which may have similarly increased the initial intracellular burden (22). Other differences in the concentration of gentamicin used or the time points that were measured may also account for the discrepancy in replication rates. For instance, we and others generally report a ratio of viable intracellular bacteria at two static time points. If intracellular bacteria are replicating at a faster rate than they are being killed by the host cell, then an overall fold increase will occur. However, if the intracellular bacteria replication rate is lower than the killing rate, then an overall fold decrease will be observed even though intracellular replication has occurred. Thus, it may be prudent to measure several different time points throughout the course of infection to determine if replication has occurred (53, 106, 137). In future, the use of a standard experimental procedure to measure AIEC intracellular survival and replication may resolve the current discrepancies.

Intracellular survival and replication is considered a crucial determinant of AIEC pathogenesis. However, this data is limited to *in vitro* studies. One unanswered question is the importance of intracellular survival and replication *in vivo*. Furthermore, there is a very limited overall understanding of AIEC-host interaction including identification of AIEC factors that promote host colonization. A mouse model in CEABAC10 mice which

express human CEACAM 3, 5, 6 and 7 receptors has been established (27, 28). In this model, mice that are infected with LF82 display clinical symptoms of colitis accompanied by a decrease in body weight and induction of IL-1β, IL-6 and IL-17 mRNA expression. LF82 is able to colonize the gut and disseminate to the liver and spleen in 20 and 27% of mice respectively. Dissemination to the liver and spleen may indicate intracellular survival *in vivo* (27). A more recent study showed that LF82 colonization increased intestinal permeability using FITC-dextran in CEABAC10 mice (41). In this model, the interaction of CEACAM6 with T1F was shown to be important for colonization. Administration of a monoclonal anti-CEACAM6 antibody significantly reduced colony forming units per gram (CFU/g) of feces on day 3 post-infection. Infection with LF82 Δ*fimH* significantly reduced CFU/g of feces by day 2 post-infection and ameliorated colitis and its associated symptoms (27). Drawbacks to this model are the use of transgenic mice limiting the study of diverse host backgrounds and significant acute mortality associated with LF82 infection limiting the study of chronic colonization. Recently, our lab established a chronic colonization model in wild-type mice including CD-1 mice and C57Bl/6 mice which mimics certain CD pathologies including intestinal inflammation and fibrosis (Submitted June 11 2012 to PLoS Pathogens). Using this model in CD-1 mice, here we showed that both NRG857c and LF82 colonization and persistence is enhanced by FimH as Δ*fimH* mutants were outcompeted by the wild-type strains in a competitive index assay. This was associated with lower bacterial burden in the feces and intestinal organs. The limit of detection for determination of WT and mutant bacteria burden via replica plating was defined as 200 CFU/mL homogenized feces or

organ since at least 20 individual colonies per 100 µL was required for consistent results. The limit of detection for total bacteria burden was defined as 10 CFU/mL homogenized feces or organ which corresponds to 1 colony per 100 µL. Data points were excluded if a fecal sample was not obtained or if the bacteria burden was below the limit of detection. If either no WT or mutant bacteria was detected, a value of one CFU was assigned for the purpose of bacteria burden and competitive index calculations. Unlike the CEABAC10 mouse model, CEACAM6 expression was not required for colonization. Interestingly, in the absence of CEACAM6, FimH was still required for colonization and persistence suggesting that there may be other ligands or receptors that FimH binds in the mouse intestine to promote colonization. FimH has been shown to bind soluble fibronectin and α3β1 integrin on the surface of bladder epithelial cells to promote cellular invasion (46, 144). Fibronectin expression is increased in the CD colon and in smooth muscle cells within CD strictures (48, 66). Numerous bacteria pathogens bind fibronectin and/or β1 integrin receptors to induce cellular invasion. For instance, *Mycobacterium avium* subspecies *paratuberculosis*, the causative agent of chronic granulomatous enteritis in ruminants, invades M cells by binding fibronectin which is bound by integrins on the surface of M cells (133). M cells uniquely express β1 integrins on their apical surface and are targeted by several gut bacteria including *Salmonella enterica* serovar Typhimurium, *Yersinia pseudotuberculosis* and AIEC (29, 33, 34). Interestingly, aphthous ulcers which are the earliest lesion in Crohn's disease form within Peyer's patches which contain M cells. Other bacteria which interact with both fibronectin and β1 integrins include *Enterococcus faecalis* (66), *Streptococcus pyogenes* (113) and *Staphylococcus aureus* (4).

Other pathogenic bacteria including Diffusely Adherent *E. coli* and *Neisseria meningitides* target α5β1 integrin receptor and CEACAM receptors to promote adhesion (102, 116, 154). Thus, targeting fibronectin, integrin receptors and CEACAM receptors is a common invasive strategy utilized by several pathogenic bacteria. Interestingly, Ordonez *et al*. demonstrated that α5β1 integrin receptor co-localizes with CEACAM5 & 6 and that binding of fibronectin to α5β1 integrin was augmented by expression of CEACAM5 & 6 (110). Taken together, it would be interesting to determine if FimH promotes AIEC colonization in wild-type mice by binding to fibronectin bound to β1 integrin receptors on the surface of M cells to promote cellular invasion and epithelial translocation. In CEABAC10 mice, this interaction would be predicted to be enhanced by the presence of CEACAM6.

Sequencing of the NRG857c and LF82 genomes has provided insight into genetic determinants that may promote host colonization and has allowed phylogenetic comparison with other pathogenic *E. coli* strains which may elucidate common colonization strategies shared by AIEC. NRG857c and LF82 have a high degree of genetic similarity and primarily differ only in their plasmid content (96, 105). Analysis of both genomes reveals unique genetic sequences which encode prophage elements and metabolic operons. However, AIEC does not possess virulence determinants typically associated with enteric *E. coli* pathogens such as *ipaC* of enteroinvasive *E. coli*, *eae* of enteropathogenic *E. coli* and enterohemorrhagic *E. coli*, Shiga-like toxin-I and –II of enterohemorrhagic hemorraghic *E. coli* and *tia* of enterotoxigenic *E. coli* (96, 105). Virulence determinants that are present include lipopolysaccharide and capsule

biosynthetic pathways, multi-drug resistance, type VI secretion systems, iron acquisition systems, metabolic operons, fimbrial operons and adhesins and invasins. Primary sequence analysis of mature FimH reveals three common missense mutations V27A, N70S and S78N and one unique LF82 mutation T158P relative to the K-12 FimH sequence. Sequence analysis of *fimH* in *E. coli* isolates from IBD patients demonstrated a significant association between A27V and a healthy individual state and between N70S and S78N and an IBD disease state (135). In a more recent study of *fimH* mutations in *E. coli* isolates from IBD and control patients, V27A and G66S were associated with a CD disease state, G66S, N70S and S78N were associated with the B2/D phylogroup and the presence of V27A, N70S and S78N together in B2 strains were associated with mild mucosal inflammation (63). V27A was shown to confer low mannose binding affinity which was associated with increased resistance to soluble inhibitors (143, 145). Variation in mannose binding affinity can alter tissue tropism and bacteria burden *in vivo* (30, 60, 145). Thus, genetic mutations in FimH and other AIEC factors may promote host colonization. Despite this genetic information, the lack of virulence determinants typically associated with enteric pathogens requires a more careful analysis of both AIEC and host factors that may facilitate chronic colonization.

Phylogenetic analysis clusters AIEC with extraintestinal pathogenic *E. coli* including uropathogenic *E. coli* (UPEC) and avian pathogenic *E. coli* (APEC). Extensive research has been done on UPEC pathogenesis including the role of FimH in adhesion, invasion and intracellular survival (46, 92, 159). An understanding of UPEC pathogenesis may elucidate common virulence strategies between UPEC and AIEC. Like AIEC, UPEC

lacks enteric virulence determinants but does encode iron acquisition systems, lipopolysaccharide and capsule biosynthetic operons, secreted toxins and several fimbrial operons (86). T1F are crucial for adherence and invasion of bladder epithelial cells as a UPEC Δ*fimH* mutant is unable to adhere to or invade bladder epithelial cells *in vitro* or colonize the bladder *in vivo* (7, 92). FimH was shown to be both necessary and sufficient for invasion of bladder epithelial cells *in vitro* (92). FimH binds α3β1 integrin on the surface of bladder epithelial cells which induces autophosphorylation of focal adhesin kinase and recruitment and activation of Src tyrosine kinase which promote endocytosis (46). Once invaded, UPEC can replicate intracellularly within superficial facet cells *in vivo* and bladder epithelial cells *in vitro* (103, 159). This intracellular population is thought to facilitate chronic persistence *in vivo* (103). Epithelial differentiation is thought to induce UPEC re-emergence and promote recurrent infection (104). Similarly, LF82 has been shown to invade and persist within intestinal epithelial cells *in vitro* (20, 137, 158). It will be interesting to determine if and where AIEC persists within the intestinal epithelium *in vivo*. Interestingly, ileal epithelial cells similarly undergo differentiation within crypts providing a possible similar environmental cue for AIEC re-emergence (37). Thus, an understanding of phylogenetically related *E. coli* pathogens may reveal certain colonization strategies employed by AIEC.

Future directions

The following research questions and experiments may help to expand upon the current data presented here.

(1) The role of the macrophage receptor CD48 in the FimH-mediated interaction

Using flow cytometric analysis, we showed that ~30% of RAW264.7 cells express CD48. To determine whether CD48 expression is critical for FimH-mediated bacteria interaction, RAW264.7 cells could be sorted by fluorescence activated cell sorting into CD48-positive and CD48-negative populations. Using a gentamicin protection assay, these distinct populations could be infected with WT or Δ*fimH* mutant bacteria and the levels of intracellular bacteria at 2 hours and total bacteria within the inoculum could be quantified. If CD48 is critical for FimH-mediated bacteria interaction, then CD48-positive cells would show an increased intracellular WT bacteria burden relative to Δ*fimH* while CD48-negative cells would show no difference between WT and Δ*fimH* intracellular burden.

(2) The role of specific FimH variants in AIEC interaction with macrophages

Analysis of the mature FimH primary sequences in NRG857c and LF82 identified four missense mutations relative to K-12: V27A, N70S, S78N and T158P where T158P is unique to LF82. Unlike NRG857c and K-12 where a consistent trend towards lower Δ*fimH* intracellular burden at 2 hours post-infection under non-opsonic conditions was observed, LF82 Δ*fimH* had a trend towards increased intracellular burden relative to LF82 in cells infected at a high MOI. To determine whether the FimH variant expressed by LF82 can account for this observation, two separate experiments can be performed. In the first experiment, allelic exchange could be used to chromosomally express different *fimH* alleles in the same background. For example, LF82 expressing NRG857c *fimH* or K-12 *fimH*, NRG857c expressing LF82 *fimH* or K-12 *fimH* or K-12 expressing NRG857c *fimH*

or LF82 *fimH* could be constructed. By using chromosomal expression versus plasmid-based expression, the effects of plasmid copy number or variable expression levels could be avoided. These strains could be used in a gentamicin protection assay and the levels of intracellular bacteria at 2 hours and total bacteria within the inoculum could be quantified. If the FimH variant affects macrophage interaction, then the phagocytic index values between an individual strain expressing different FimH variants would differ and could be specifically attributed to the FimH variant being expressed. In the second experiment, purified FimHC from NRG857c, LF82 or K-12 could be coated on fluorescent microspheres and phagocytosis could be monitored by confocal fluorescence microscopy. Differences in the numbers of adherent or intracellular beads could be specifically attributed to the FimHC variant coated on the beads. These experiments may help to determine whether the FimH variants expressed by each *E. coli* strain affect interaction with macrophage cells.

(3) The role of intracellular survival and replication within macrophages *in vivo*

We were unable to observe extensive AIEC replication within macrophages *in vitro* which is inconsistent with some, but not all of the published literature (13, 21, 22, 23, 53, 148). To determine whether AIEC survival and replication within macrophages is important *in vivo*, wild-type mice could be infected with AIEC expressing chromosomally encoded green fluorescent protein (GFP) and AIEC interaction with macrophages could be monitored over the course of the infection. More specifically, the cecum and colon could be harvested at various time points during the infection and subjected to either flow cytometric or immunofluorescence analysis. Using flow cytometry, lamina propria

macrophages within the cecum or colon could be labelled with the F4/80 macrophage marker and assessed for GFP expression. GFP signal from extracellular bacteria could be quenched using trypan blue so that only the intracellular GFP signal is measured. This signal should be normalized to the number of macrophages to account for variation in the number of lamina propria macrophages. If GFP-AIEC survives or replicates within macrophages *in vivo*, then the number of macrophages that are GFP-positive should remain the same or increase over the course of the infection. In contrast, if the number of GFP-positive macrophages decreases, then it is likely that GFP-AIEC is killed intracellularly. Using immunofluorescence, cecal and colonic sections could be treated with gentamicin to kill extracellular bacteria prior to fixation and staining with anti-F4/80 and anti-GFP primary antibodies and fluorescent secondary antibodies. This would provide direct visualization of intracellular GFP-AIEC. If GFP-AIEC survives or replicates within macrophages, then the number of macrophages that contain intracellular GFP-AIEC should increase over the course of the infection. Together, flow cytometric and immunofluorescence analysis should provide evidence for AIEC intracellular survival or replication within macrophages *in vivo*.

(4) Evaluating the role of β1 integrin and/or fibronectin in FimH-mediated colonization

To initially determine whether β1 integrin and/or fibronectin is involved in FimH-mediated AIEC colonization *in vivo*, CD-1 mice could be infected with GFP-labelled WT or Δ*fimH* bacteria. After colonization has been established, infected cecal and colonic tissues could be isolated, fixed and stained with anti-β1 integrin, anti-fibronectin and anti-GFP antibodies. If either or both β1 integrin and fibronectin is required for colonization,

then WT bacteria should have increased co-localization relative to Δ*fimH* bacteria. To more specifically study this interaction, a gut loop assay could be employed as outlined in (133). Briefly, ileocecal loops were injected with either bacteria only, fibronectin-opsonised bacteria only, fibronectin-opsonised bacteria and fibronectin blocking peptides or fibronectin-opsonised bacteria and anti-β1 integrin antibody. After 2 hours, gut loops were isolated, fixed and examined by immunofluoresence microscopy. By counting the number of infected cells, the authors were able to determine if fibronectin and β1 integrin enhanced intestinal colonization. A similar experimental procedure could be used to determine whether AIEC utilizes fibronectin or β1 integrin receptors to promote colonization.

Conclusion

The CD inflammatory microenvironment represents a unique niche that favors AIEC colonization. Our data shows that FimH is important for colonization and persistence within the murine intestine *in vivo* and may contribute to macrophage interaction *in vitro*. Although we were unable to demonstrate extensive replication within macrophages *in vitro*, it remains to be determined whether macrophages provide a replicative or persistent niche *in vivo*. Further development and characterization of mouse models of AIEC colonization will deepen our understanding of AIEC-host interaction. Although AIEC does not likely represent the sole microbial trigger in CD, an understanding of its role in CD etiopathogenesis will provide a solid foundation for

characterizing the complex interaction between the gut microflora, immune response and genetic factors in CD.

Experimental Procedures

Construction of *fimH* mutant strains

fimH was deleted in NRG857c, LF82 and K-12 using the Lambda-Red recombinase method (40). Briefly, chloramphenicol or kanamycin resistance cassettes were amplified from pKD3 or pKD4 respectively using the primers AIECfimHfwd and AIECfimHrev for NRG857c and LF82, or K12fimHfwd and K12fimHrev for K-12. The PCR product was digested with DpnI (New England Biolabs, Canada) and transformed into chemically competent cells containing pKD46. After recovery at 30°C, cells were plated on selective media and incubated overnight at 30°C. Positive clones were confirmed using the primers fimHscrfwd2 and either AIECfimHrev2 or K12fimHrev2.

Construction of FimH complementation vectors

fimH was PCR amplified from NRG857c, LF82 and K-12 genomic DNA using the primers BluefimHfwd and BluefimHrev and PCR cycling conditions: 95°C 1 minute, 53°C 1 minute, 68°C 1 minute. The PCR product and arabinose-inducible cloning vector pBADGr (51, 83) were digested with EcoRI (New England Biolabs, Canada) and XbaI (New England Biolabs, Canada), ligated together, and transformed into chemically competent DH5α cells. Clones were selected on gentamicin (50 μg/mL) and sequenced using the primers BADF and BADR.

Type 1 fimbriae inducing conditions

Bacteria were statically grown in 3 mL of Luria-Bertani (LB) broth with antibiotics at 37°C for 24 hours, diluted 1:1000 into 3 mL of LB broth with antibiotics, and statically grown at 37°C for an additional 24 hours (adapted from (30)). For mouse infections, the volume of LB broth was increased to 15 mL for the 1:1000 dilution.

Yeast agglutination assay

Bacteria were grown under type 1 fimbriae inducing conditions. 2-3 mL of resuspended overnight culture was spun down at 4000 x *g* for 2 minutes at room temperature, washed twice in phosphate buffered saline (PBS) and resuspended to an OD_{600} ~ 1 (equal to ~$2x10^9$ cfu/mL) in 100 µL of PBS. To inhibit agglutination, 0.25g of D-mannose (Bioshop, Canada) was suspended in 10mL of PBS and filter-sterilized. 25 µL of 2.5% (w/v) D-mannose solution was added to each well in a 96-well U-bottom suspension culture plate (Cellstar 650185, Grenier bio-one). 25 µL of bacteria was added to the first well and incubated for 10 minutes at room temperature. Bacteria were then serially diluted and incubated for an additional 10 minutes at room temperature. To measure agglutination, 25 µL of bacteria was serially diluted in PBS and 25 µL of a 2% (w/v) baker's yeast solution was then added to all wells. The bacteria-yeast suspension was gently rotated at 90 rpm for 10 minutes at room temperature. The last well to yield a positive agglutination reaction was recorded (62, 73).

Flow cytometric analysis of CD48 expression on the surface of RAW264.7 macrophage-like cells

RAW264.7 cells were collected and re-suspended at $1x10^7$ cells/mL in FACS buffer (0.2% BSA, PBS). 100 µL of cells was added to a U-bottom 96-well plate and centrifuged at 500 x g for 5 minutes at 4°C. The supernatant was removed and 100 µL of 1/100 dilution of anti-mouse CD16/32 was added. After incubation on ice for 15 minutes, cells were centrifuged using the same parameters and the supernatant was removed. 100 µL of 1/400 dilution of anti-CD48 or isotype control or 100 µL of FACS buffer was added. After incubation on ice for 30 minutes in the dark, cells were centrifuged and the supernatant was discarded. Cells were washed two times with 200 µL of FACS buffer, resuspended in 200 µL of fixation buffer (2% paraformaldehyde, PBS) and stored at 4°C. Prior to FACS analysis, cells were resuspended and added to a cell strainer cap. Flow cytometry tubes were centrifuged at 1000 rpm for 3 minutes at 4°C. Samples were read on the LSR II flow cytometer (BD Biosciences).

Gentamicin protection assay

RAW264.7 macrophage-like cells (119) and J774-A1 macrophage-like cells (118) were maintained in Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal bovine serum (FBS) + 50 units/mL penicillin-streptomycin at 37°C and 5% CO_2. RAW264.7 cells were seeded at $5x10^5$ cells/mL in 24-well plates or J774-A1 cells were seeded at $5x10^4$ cells/200 µL in 96-well plates in DMEM + 10% FBS 24 hours prior to infection. Bacteria grown under T1F inducing conditions were spun down at 4000 x *g* for 2 minutes

at room temperature, washed twice in PBS and resuspended in 1 mL of PBS. Macrophage-like cells were washed three times in PBS and infected at a MOI of ~10 or 100. Cells were spun at 500 x *g* for 5 minutes at room temperature to synchronize the infection and incubated at 37°C, 5% CO_2 for 30 minutes. Cells were washed three times in PBS and incubated with DMEM + 10% FBS containing 100 µg/mL gentamicin for one hour. For the 2h time point, cells were washed three times in PBS and lysed in either 250 µL of lysis buffer (1% Triton-X-100, 0.1% SDS, PBS) in 24-well plates or 50 µL of lysis buffer in 96-well plates for 10-15 minutes at 37°C, 5% CO_2. For the 20h time point, cells were washed three times in PBS and incubated with DMEM + 10% FBS containing 10 µg/mL gentamicin. At 20h, cell supernatants were collected and cells were washed three times in PBS and lysed. Bacteria were serially diluted in PBS and 10 µL was spotted in triplicate on selective media for enumeration. Phagocytic index was calculated as Intracellular bacteria levels at 2h / Initial inoculum. Fold survival was calculated as Intracellular bacteria levels at 20h / Intracellular bacteria levels at 2h (65).

Opsonisation of bacteria

Bacteria grown under T1F inducing conditions were spun down at 4000 x *g* for 2 minutes at room temperature, washed twice in PBS and resuspended in 1 mL of PBS. 250 µL of bacteria was added to 750 µL of PBS for OD_{600} determination. The remaining 750 µL of bacteria was spun down and resuspended in opsonisation media (20% normal human serum in DMEM + 10% FBS). Bacteria were incubated at 37°C for 30 minutes in a water bath and resuspended in PBS.

TNF-α enzyme-linked immunosorbent assay

A 96-well high-binding flat-bottom microplate (Corning) was coated with 100 µL per well of capture antibody, diluted to 0.8 µg/mL in PBS and incubated overnight at room temperature. The plate was washed three times with wash buffer (0.05% Tween 20 in PBS), blocked with 300 µL of reagent diluent (1% bovine serum albumin in PBS, 0.2 µm-filtered) for one hour at room temperature and then washed three times with wash buffer. Cell supernatant samples were diluted in reagent diluent and 100 µL of sample was added to each well and incubated for two hours at room temperature. The plate was washed three times with wash buffer and incubated with 100 µL of detection antibody, diluted to 200 ng/mL in reagent diluent, per well for two hours at room temperature. The plate was washed three times with wash buffer and incubated with 100 µL of streptavidin-HRP, diluted 1:200 in reagent diluent, for 20 minutes at room temperature in the dark. The plate was washed three times with wash buffer and incubated with 100 µL of substrate solution per well for 10 minutes at room temperature in the dark. 50 µL of 2N H_2SO_4 was added to each well to stop the reaction, and the absorbance was read at 450 nanometres.

Animal experiments: Competitive index assay

Wild-type and Δ*fimH* mutant bacteria were statically grown in 3mL LB + antibiotic at 37°C for 24 hours, diluted 1:1000 into 15 mL LB + antibiotic in a 125-mL flask and incubated for 22 hours under static conditions at 37°C. $1x10^{10}$ colony-forming units were spun down (4000 x g, 2 minutes), washed two times in PBS and resuspended

in 500 µL of PBS. Wild-type and Δ*fimH* mutant bacteria were mixed in a 1:1 ratio for a final inoculum concentration of ~$1x10^{10}$ cfu/mL. Female CD-1 mice (Charles River Laboratories), age 6-8 weeks, were pre-treated with 20 mg of streptomycin 24 hours prior to infection and infected with either 100 µL of inoculum or PBS (day 0). Body weight was monitored and fecal pellets were collected every 2-3 days. Fecal pellets were weighed, homogenized in 1mL PBS by vortexing and serially diluted in PBS. NRG857c and NRG857c Δ*fimH* bacteria were selected on chloramphenicol (34 µg/mL) streptomycin (100 µg/mL) plates. LF82 and LF82 Δ*fimH* bacteria were selected on ampicillin (200 µg/mL) plates. NRG857c and NRG857c Δ*fimH* bacteria were replica plated onto chloramphenicol (34 µg/mL) streptomycin (100 µg/mL) and chloramphenicol (34 µg/mL) streptomycin (100 µg/mL) kanamycin (50 µg/mL) plates. LF82 and LF82 Δ*fimH* bacteria were replica plated onto ampicillin (200 µg/mL) and chloramphenicol (34 µg/mL) plates and enumerated. Competitive index values were determined by $[(\Delta fimH \text{ cfu / Wild-type cfu})]_{output}$ / $(\Delta fimH \text{ cfu / Wild-type cfu})]_{input}$.

Figures and Figure Legends

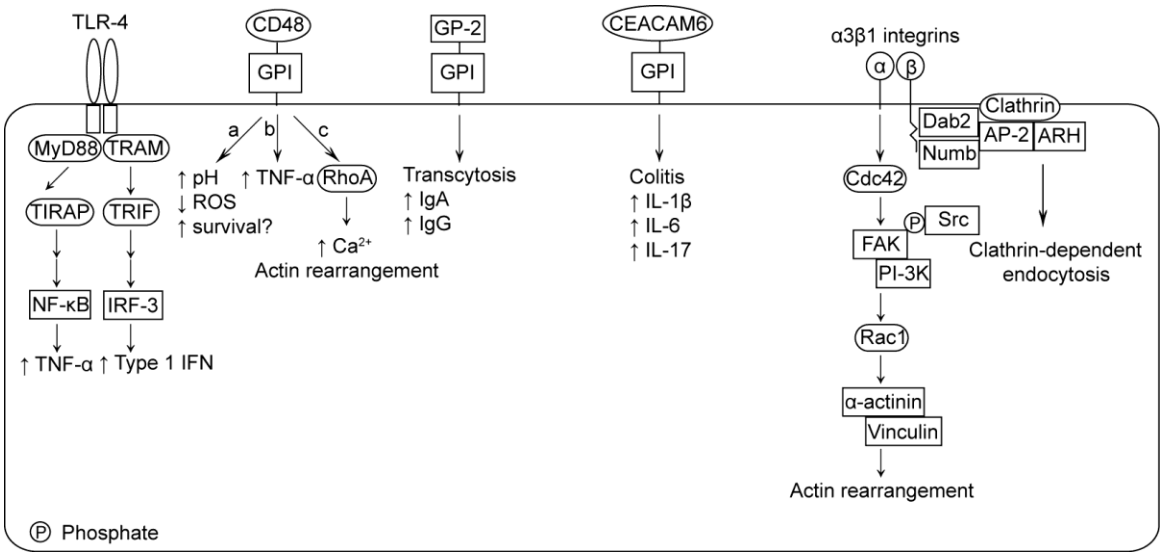


Figure 1. The mannose binding adhesin FimH binds an array of receptors on different cell types. From the left, FimH binds the innate immune receptor TLR-4 to induce MyD88-dependent and TRIF/TRAM-dependent signaling. FimH interacts with GPI-anchored CD48 on macrophages which increased intracellular pH and decreased reactive oxygen species (ROS) which may contribute to enhanced survival (a), mast cells which induced TNF-α release (b), and endothelial cells which activated RhoA and increased cytosolic calcium levels which are implicated in actin rearrangement (c). In the gut, FimH binds GP-2 on M cells which was required for transcytosis and activation of an adaptive immune response and CEACAM6 on ileal epithelial cells which induced colitis and upregulated mRNA levels of the pro-inflammatory cytokines IL-1β, IL-6 and IL-17. In the bladder, binding of α3β1 integrins on bladder epithelial cells activates the GTPase Cdc42, autophosphorylation of focal adhesin kinase (FAK), recruitment of Src tyrosine

kinase and phosphoinositide-3kinase (PI-3K), activation of the GTPase Rac1, and association of the actin adaptor proteins α-actinin and vinculin leading to actin rearrangement. Entry via clathrin-dependent endocytosis requires clathrin, adaptor protein complex-2 (AP-2) and alternate endocytic adaptors Numb, Dab2 and ARH.

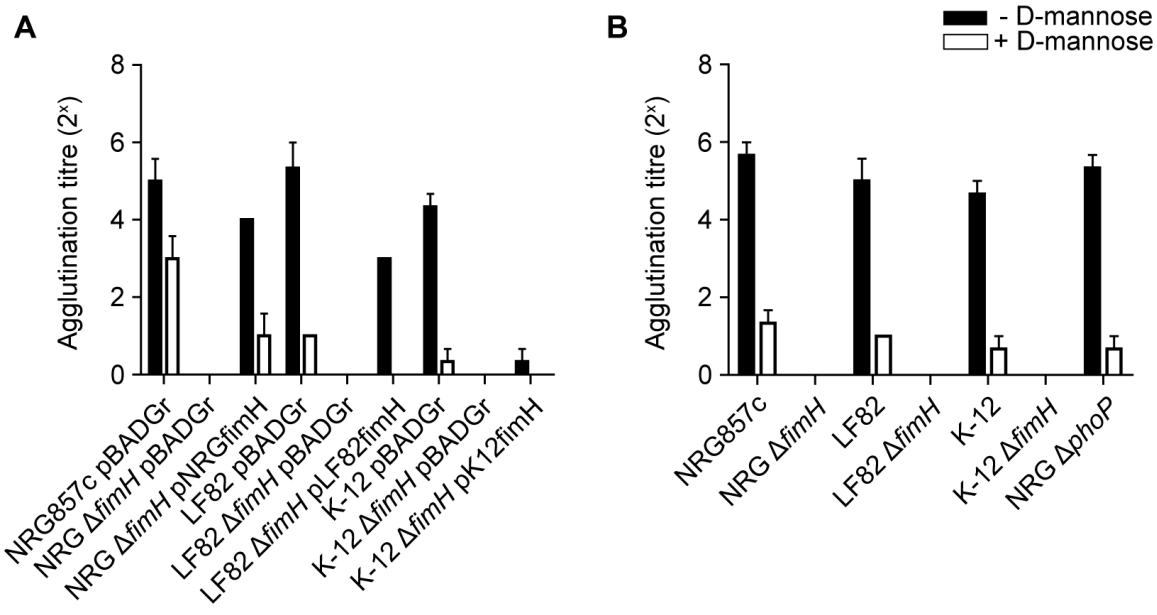


Figure 2. Type 1 fimbriae are expressed under static conditions. Type 1 fimbriae expression in wild-type and mutant strains with (A) and without an empty pBADGr vector (B) was semi-quantitatively measured using a yeast agglutination assay in the absence (black bars) or presence (white bars) of 2.5% (w/v) D-mannose. The pBADGr expression vector carrying a wild-type *fimH* copy was used to complement Δ*fimH* mutant strains (A). Yeast agglutination titers correspond to the last positive agglutination reaction. Data represents mean + SEM, n = 3.

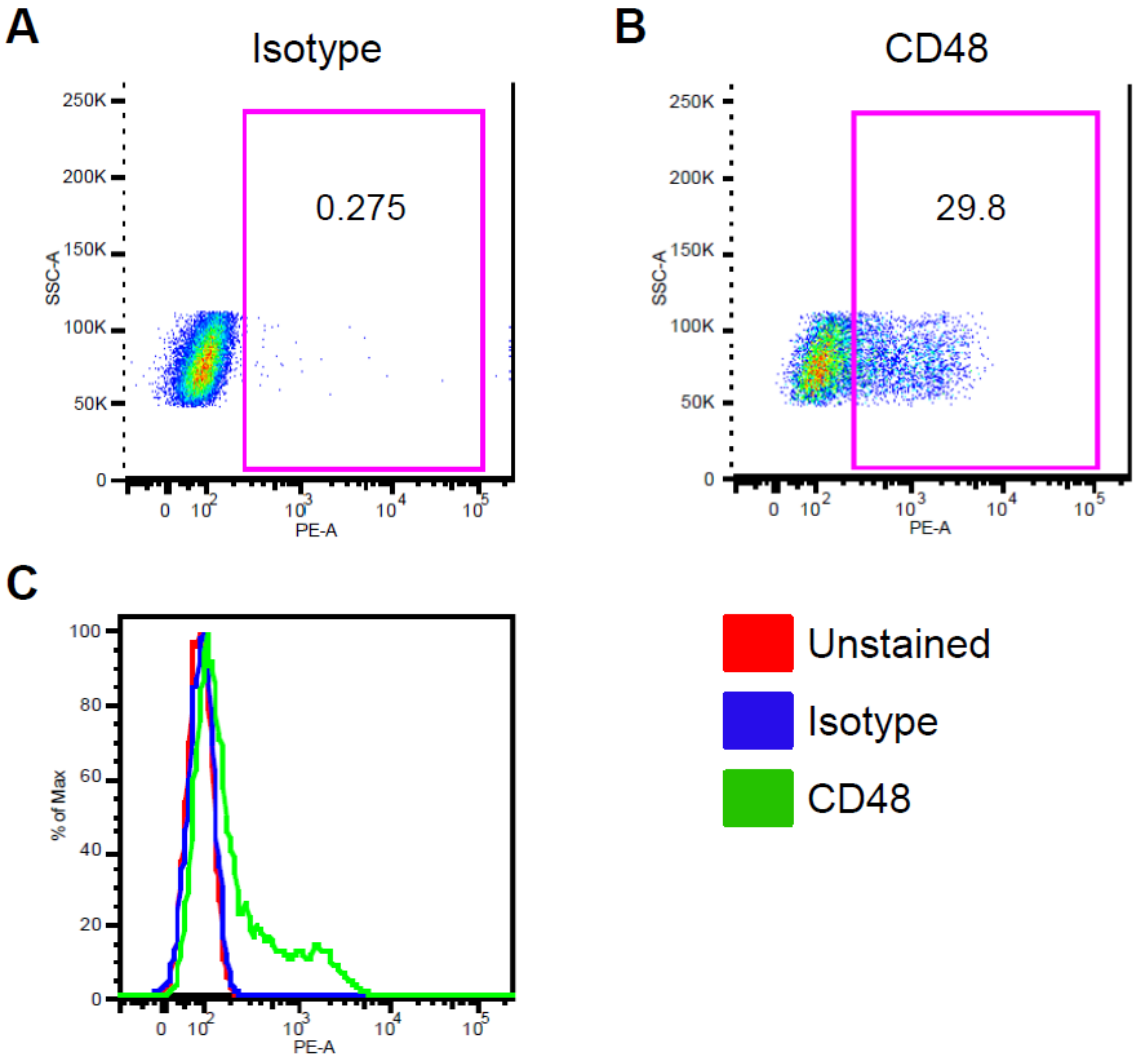


Figure 3. RAW264.7 cells express the FimH receptor CD48. CD48 expression on the surface of RAW264.7 cells was analyzed by flow cytometry. RAW264.7 cells were stained with either an isotype control antibody (A) or PE anti-mouse CD48 antibody (B). Unstained cells and isotype control antibody-stained cells were used as controls for non-specific staining (A&C). Data was analyzed using FlowJo flow cytometry analysis software.

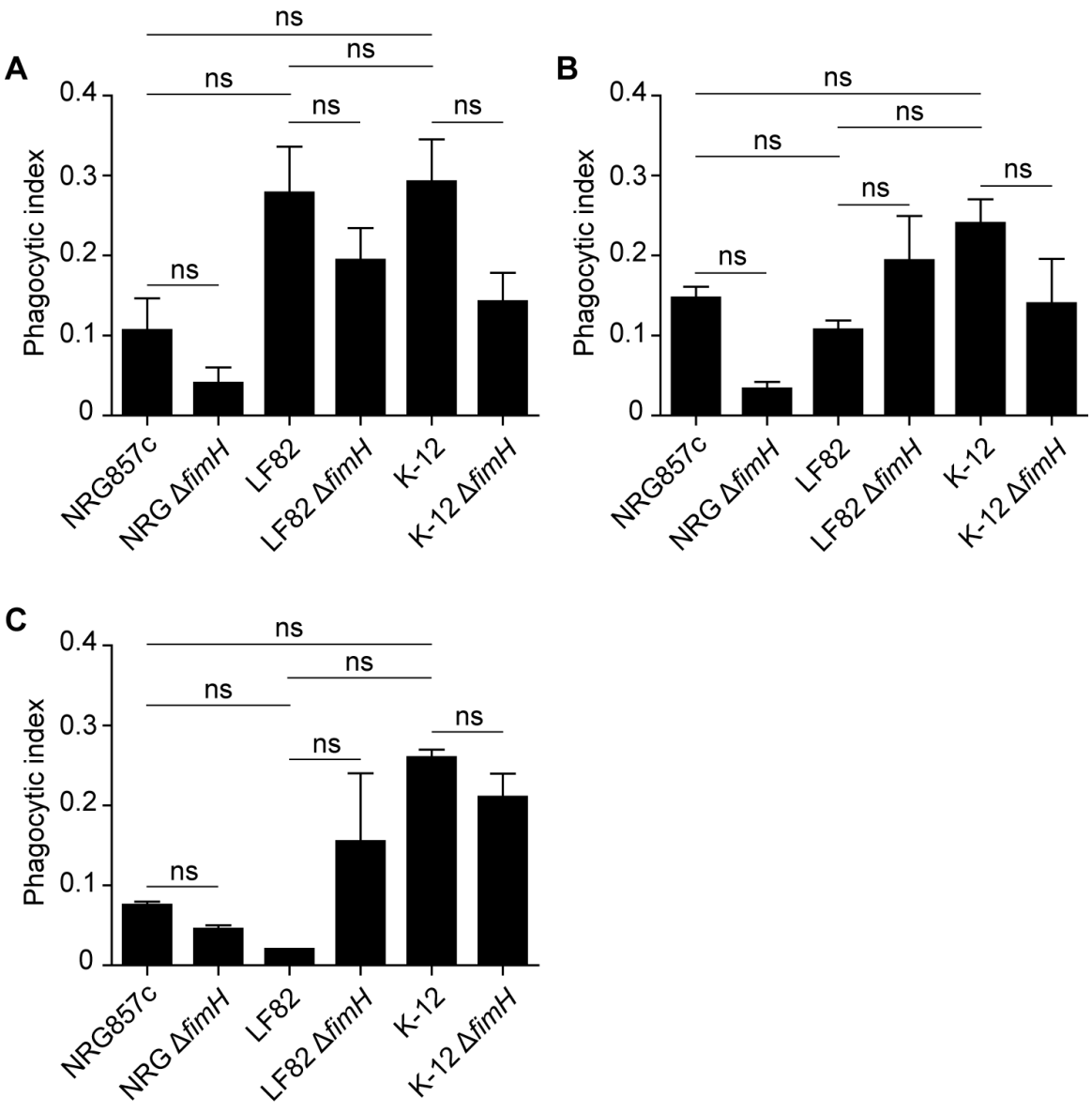


Figure 4. Loss of FimH may reduce intracellular bacteria numbers at 2 hours post-infection. RAW264.7 cells were infected with wild-type or Δ*fimH* mutant strains at a MOI of 10 (A) or 100 (B). J774-A1 cells were infected at a MOI of 100 (C). Phagocytic index representing levels of viable intracellular bacteria at 2 hours post-infection relative to the starting inoculum are expressed as the mean with standard errors from three

technical replicates from three to five experiments. Statistical significance was measured using a non-parametric one-way ANOVA statistical test ($p>0.05$, ns).

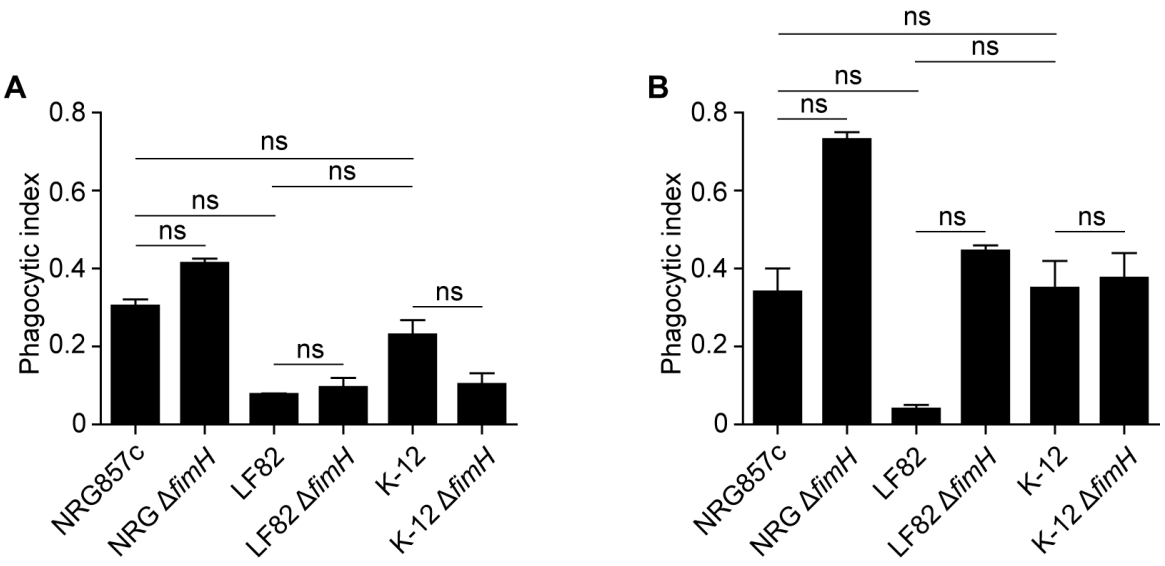


Figure 5. Bacteria opsonisation compensates for the loss of FimH. RAW264.7 cells (A) and J774-A1 cells (B) were infected with serum opsonised wild-type or Δ*fimH* mutant strains at a MOI of 100. Phagocytic index representing levels of viable intracellular bacteria at 2 hours post-infection relative to the starting inoculum are expressed as the mean with standard errors from three technical replicates from two to three experiments. Statistical significance was measured using a non-parametric one-way ANOVA statistical test ($p>0.05$, ns).

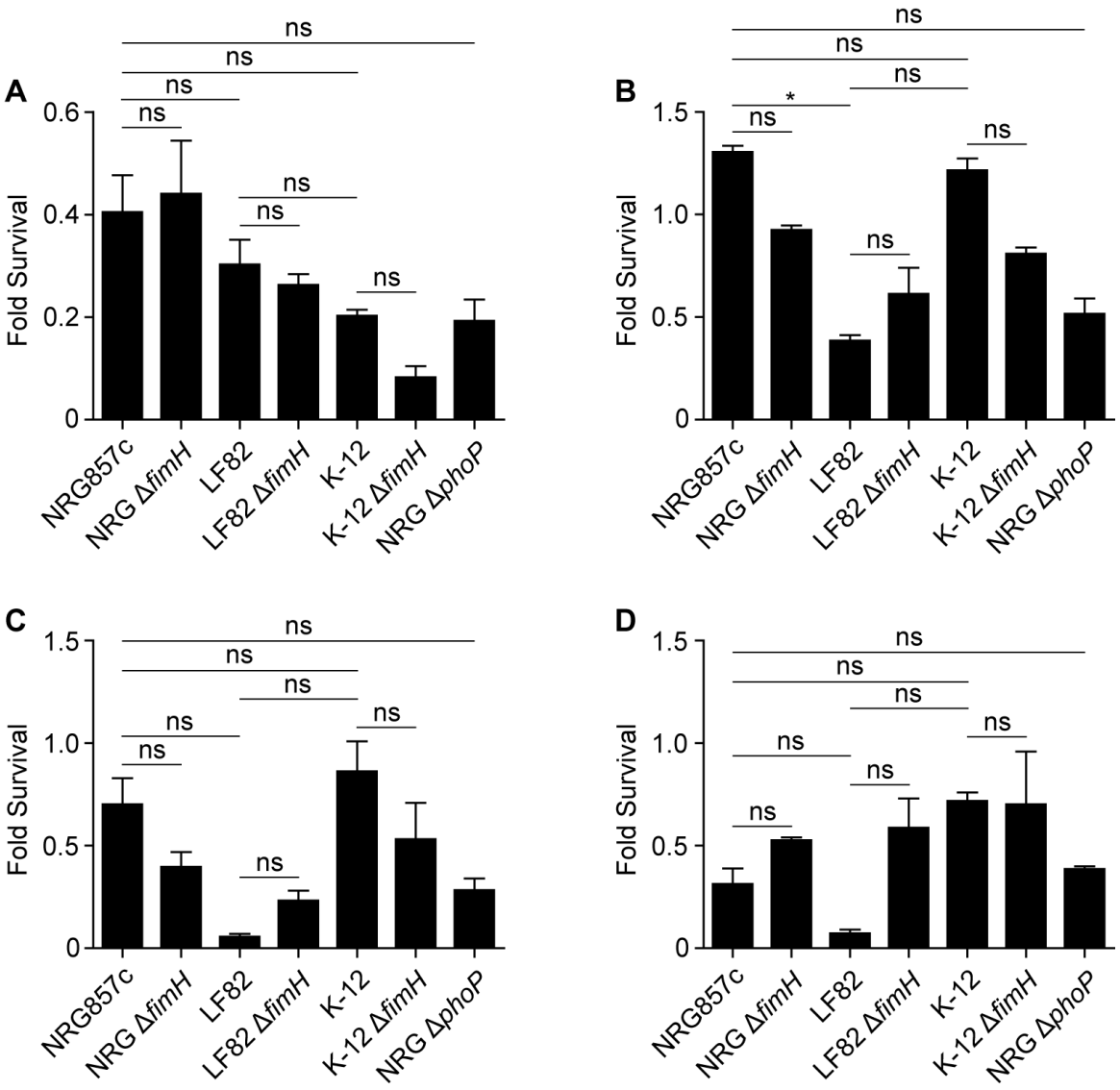


Figure 6. FimH may contribute to intracellular survival. RAW264.7 cells were infected with unopsonised bacteria at an MOI of 10 (A) or opsonised bacteria at an MOI of 100 (B). J774-A1 cells were infected at an MOI of 100 with either unopsonised (C) or opsonised bacteria (D). Fold survival representing levels of viable intracellular bacteria at 20 hours post-infection relative to 2 hours post-infection are expressed as the mean with standard errors from three technical replicates from two to three experiments. Statistical

significance was measured using a non-parametric one-way ANOVA statistical test (* indicates $p<0.05$, ns indicates $p>0.05$).

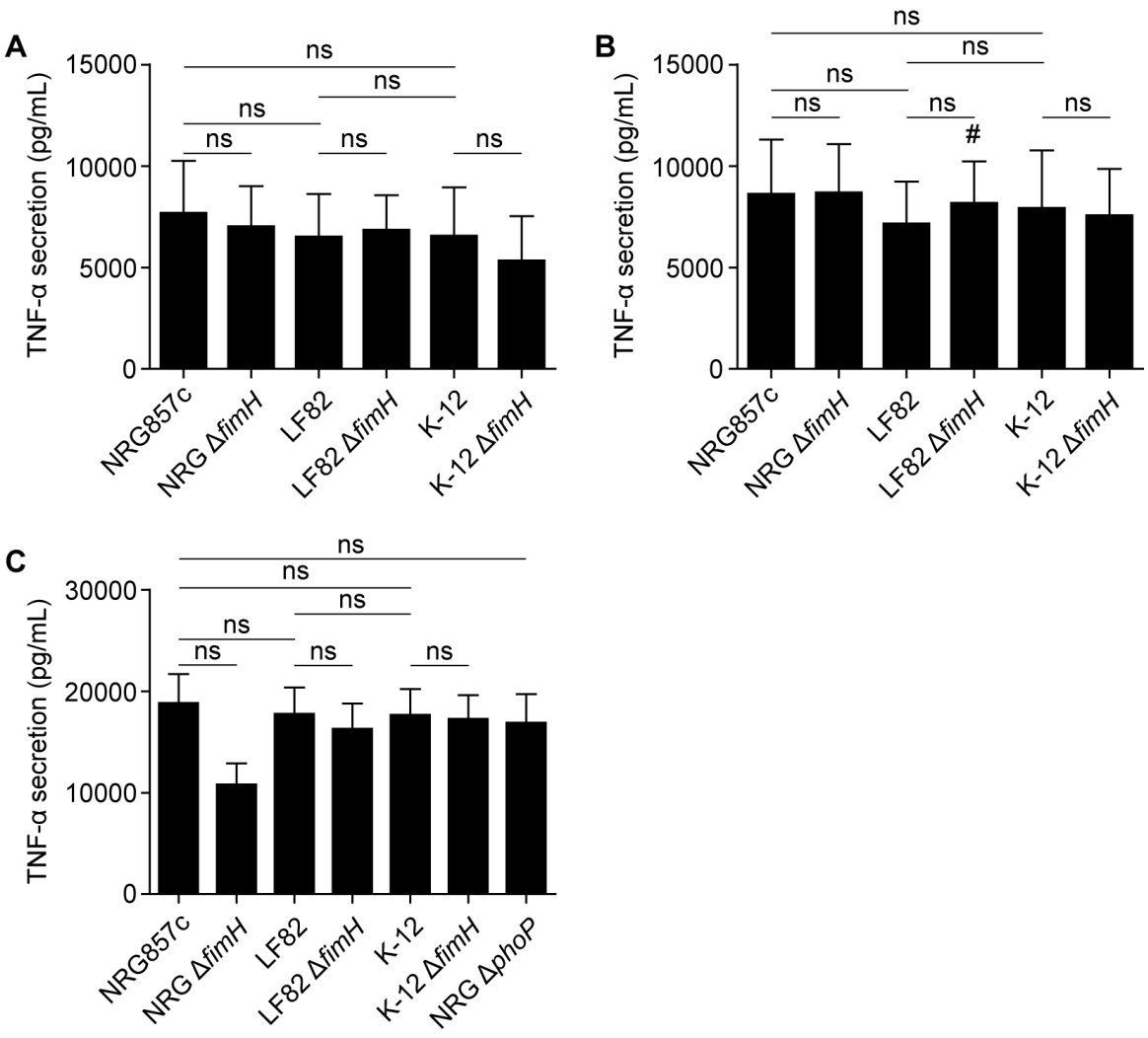
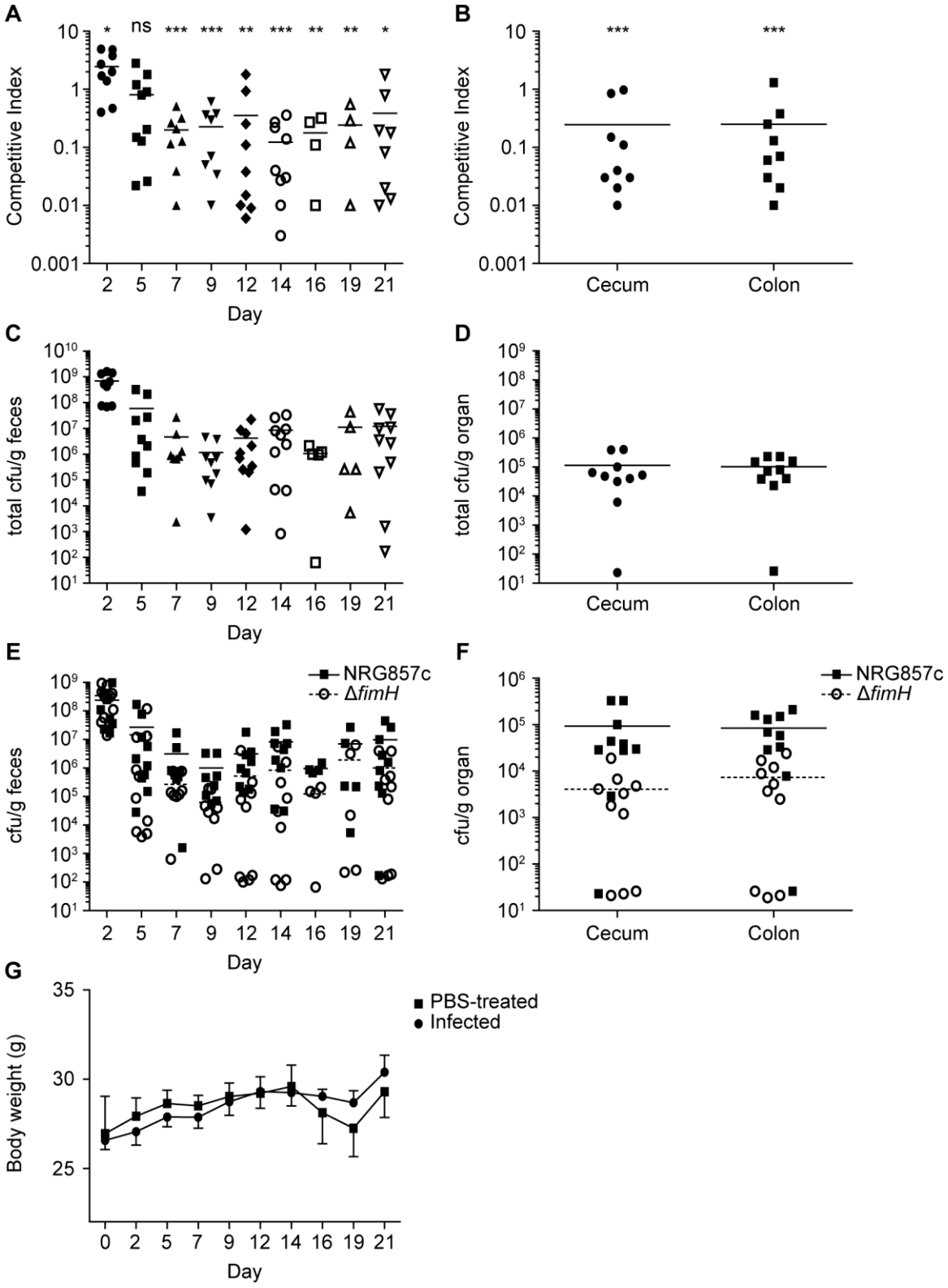


Figure 7. FimH does not affect tumour necrosis factor alpha secretion. RAW264.7 cells were either PBS-treated or infected at an MOI of 100 with unopsonised (A) or opsonised (B) bacteria or at an MOI of 10 with unopsonised bacteria (C). Cell supernatants were collected at 2 (A&B) or 20 hours post-infection (C). TNF-α levels were determined by ELISA and expressed as the mean with standard errors from three technical replicates from three to five experiments. # indicates MOI < 100. Statistical

significance was measured using a non-parametric one-way ANOVA statistical test ($p>0.05$, ns).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Body weight (g)
PBS-treated
Infected
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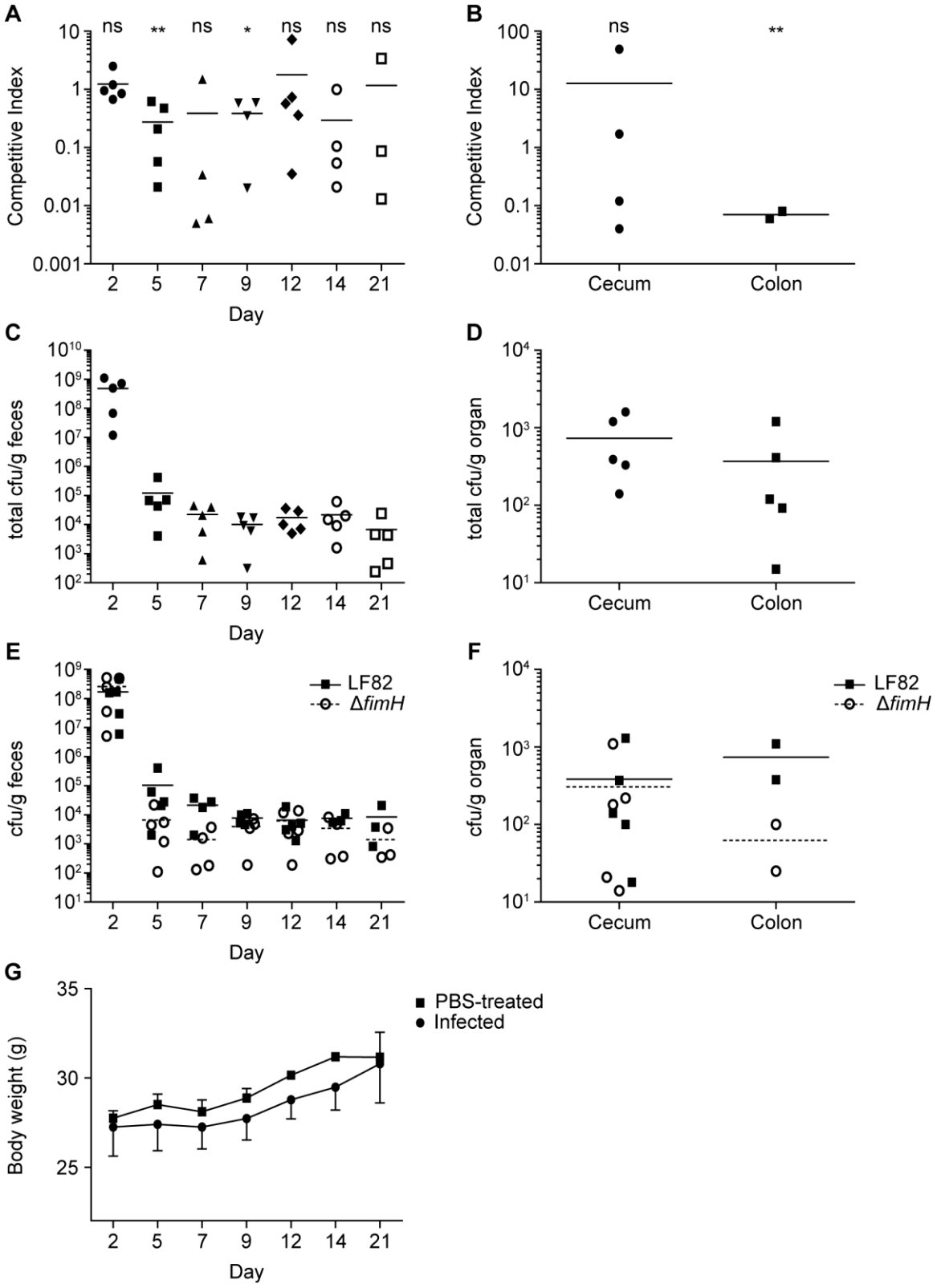
Figure 8. FimH contributes to persistence of NRG857c in CD-1 mice. Female CD-1 mice were orally infected with a 1:1 inoculum of NRG857c and NRG857c Δ*fimH* or PBS on day 0. Bacteria colonization was monitored by fecal output (A, C, E) and intestinal organ burden (B, D, F). Body weight of infected (circle) and PBS-treated (square) mice was monitored over the course of infection and expressed as the mean with standard error (G). Competitive indices from the feces (A) and intestinal organs (B), total cfu counts in the feces (C) and intestinal organs (D) and relative levels of NRG857c (black square, solid line) and NRG857c Δ*fimH* (open circle, dotted line) in the feces (E) and intestinal organs (F) were determined. Horizontal lines represent the experimental mean. Data points were excluded if a fecal sample was not collected or if the bacteria burden was below the limit of detection. Data represents two independent experiments with five mice per experiment. Data from one independent experiment is shown on day 16 and 19. Statistical significance was measured using a one-sample t test (*** indicates $p<0.001$, ** indicates $p<0.01$, * indicates $p<0.05$, ns indicates not significant).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LF82
ΔfimH
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G
Body weight (g)
PBS-treated
Infected
Day

Figure 9. FimH contributes to persistence of LF82 in CD1 mice. Female CD-1 mice were orally infected with a 1:1 inoculum of LF82 and LF82 Δ*fimH* or PBS on day 0. Bacteria colonization was monitored by fecal output (A, C, E) and intestinal organ burden (B, D, F). Body weight of infected (circle) and PBS-treated (square) mice was monitored over the course of infection and expressed as the mean with standard error (G). Competitive indices from the feces (A) and intestinal organs (B), total cfu counts in the feces (C) and intestinal organs (D) and relative levels of LF82 (black square, solid line) and LF82 Δ*fimH* (open circle, dotted line) in the feces (E) and intestinal organs (F) were determined. Horizontal lines represent the experimental mean. Data points were excluded if a fecal sample was not collected or if the bacteria burden was below the limit of detection. Data represents one independent experiment with five mice. Statistical significance was measured using a one-sample t test (** indicates $p<0.01$, * indicates $p<0.05$, ns indicates not significant).

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Appendix

List of Strains Made

NRG857c pBADGr...BKC 31-59, 60
NRG857c Δ*fimH*...BKC 31-49, 50
NRG857c Δ*fimH* clone B (not used in experiments)...BKC 31-51, 52
NRG857c Δ*fimH* pBADGr...BKC 31-61, 62
NRG857c Δ*fimH* pNRGfimH...BKC 31-63, 64
NRG857c Δ*fimH* pLF82fimH...BKC 31-65, 66
NRG857c Δ*fimH* pK12fimH...BKC 31-67, 68
LF82 pBADGr...BKC 31-69, 70
LF82 Δ*fimH*...BKC 31-53, 54
LF82 Δ*fimH* pBADGr...BKC 31-71, 72
LF82 Δ*fimH* pNRGfimH...BKC 31-73, 74

LF82 Δ*fimH* pLF82fimH...BKC 31-75, 76
LF82 Δ*fimH* pK12-fimH...BKC 31-77, 78
K-12 pBADGr...BKC 32-2, 3
K-12 Δ*fimH*...BKC 31-55, 56
K-12 Δ*fimH* pBADGr...BKC 32-4, 5
K-12 Δ*fimH* pNRGfimH...BKC 32-6, 7
K-12 Δ*fimH* pLF82fimH...BKC 32-8, 9
K-12 Δ*fimH* pK12fimH...BKC 32-10, 11
DH5α pNRGfimH...BKC 32-12, 13
DH5α pLF82fimH...BKC 32-14, 15
DH5α pK12fimH...BKC 32-16, 17
DH5α λ pir pRE112-Gen...BKC 32-24, 25
DH5α pNRGFimHC-His...BKC 32-18, 19
DH5α pLF82FimHC-His...BKC 32-20, 21
DH5α pK12FimHC-His...BKC 32-22, 23
DH5α pNRGFimC-His
DH5α pK12FimC-His
DH5α pNRGFimC-GGA-His

List of Plasmids Made

pNRGfimH
: NRG857c *fimH* cloned into MCS of pBADGr

pLF82fimH
: LF82 *fimH* cloned into MCS of pBADGr

pK12fimH
: K-12 *fimH* cloned into MCS of pBADGr

pRE112-Gen
: pRE112 encoding the gentamicin resistance cassette from pUCGm

pNRGFimHC-His
: NRG857c *fimH* cloned into MCS1 and NRG857c *fimC*-His cloned into MCS2 of pCOLADuet-1 (Novagen)

pLF82FimHC-His
: LF82 *fimH* cloned into MCS1 and NRG857c *fimC*-His cloned into MCS2 of pCOLADuet-1

pK12FimHC-His
: K-12 *fimH* cloned into MCS1 and K-12 *fimC* cloned into MCS2 of pCOLADuet-1

pNRGFimC-His
: NRG857c *fimC*-His cloned into MCS2 of pCOLADuet-1

pK12FimC-His
: K-12 *fimC*-His cloned into MCS2 of pCOLADuet-1

pNRGFimC-GGA-His
: NRG857c truncated *fimC*-His lacking the N-terminal periplasmic signal sequence cloned into MCS2 of pCOLADuet-1

List of Primers

Construction of ΔfimH *strains using Lambda Red recombination* (40)

AIECfimHfwd 5' – ACAGCTGAACCCGAAGAGATGATTGTAATGAAACGAGTTGTGTA – 3'
AIECfimHrev 5' – ACCTGCATTAGCAATGCCCTGTGATTTCTTTATTGATAAACCATA – 3'
K12fimHfwd 5' – ACAGCTGAACCCGAAGAGATGATTCTAATGAAACGAGTTGTGTA – 3'
K12fimHrev 5' – ACCAGCATTAGCAATGTCCTGTGATTTCTTTATTGATAAACCATA – 3'

Screening ΔfimH *mutant strains*

fimHscrfwd2 5' – GTGGCAACACATTGAATACTGG – 3'
AIECfimHrev2 5' – TCGGATTGTCGGTAAAGTGC – 3'
K12fimHrev2 5' – GGAACAGACCAGCAAAGTGC – 3'

Screening fimH *in CDEC isolates*

AIECfimHscrfwd 5' – GCTGAACCCGAAGAGATG – 3'
AIECfimHscrrev 5' – GCAATGCCCTGTGATTTCTTT – 3'

Construction of fimH *complementation vectors in* pBADGr

BluefimHfwd 5' – GTCCGAATTCATGAAACGAGTTATTACCCTGTT – 3'
BluefimHrev 5' – ATCCTCTAGATTATTGATAAACAAAAGTCACGC – 3'

Screening fimH *complementation vectors*

BADF 5' – AAGTGTCTATAATCACGGCAGA – 3'
BADR 5 – TCACTTGAGTTCGGCTGG – 3'

Construction of pRE112-Gen

pUCGmF-2 5'- ATGCGGGCCCATTGACATAAGCCTGTTCGGTT – 3'
pUCGmR 5' – GATCGGGCCCTTAGGTGGCGGTACTTGGGTC – 3'

Construction of FimH-FimC-His purification vectors in pCola-Duet

Primers to amplify *fimH* to clone into MCS1 minus N-terminal His tag

fimHpurFNcoI 5' – ATGCCCATGGGCATGAAACGAGTTATTACCCTGTT – 3'
DuetfimHrev 5' – ATCCGTCGACTTATTGATAAACAAAAGTCACGC – 3'

Primers to amplify *fimC* with a C-terminal His tag to clone into MCS2

fimCpurF 5' – GCTTATCCAGATCTAGTGAGTAATAAAAACGTCAATGTAAG – 3'
fimCHispurR 5' – ATGCCTCGAGTTAGTGATGGTGATGGTGATGTTCCATTACGCCCGTCATTTT – 3'

Primers to amplify *fimC* with a C-terminal His tag minus periplasmic signal sequence to clone into MCS2

fimCpurfwdGGA: 5’ – GCTTATCCAGATCTAGGAGTGGCCTTAGGTGCGACT – 3’
fimCHispurR: 5’ – ATGCCTCGAGTTAGTGATGGTGATGGTGATGTTCCATTACGCCCGTCATTTT – 3’

Screening FimH-FimC-His purification vectors
Multiple cloning site 1
DuetUP1 5’ – GGATCTCGACGCTCTCCCT – 3’
DuetDOWN1 5’ – GATTATGCGGCCGTGTACAA – 3’

Multiple cloning site 2
DuetUP2 5’ – TTGTACACGGCCGCATAATC – 3’
DuetDOWN2 5’ – GCTAGTTATTGCTCAGCGG – 3’

Sequencing fimH *in CDEC isolates*
AIECfimHscrfwd 5’ – GCTGAACCCGAAGAGATG – 3’
AIECfimHscrrev 5’ – GCAATGCCCTGTGATTTCTTT – 3’

Construction of fimH *allelic exchange vectors* (primer design only)
Amplification of *fimH*
fimHF 5’ – ATGAAACGAGTTATTACCCTGTT – 3’
fimHR 5’ – TTATTGATAAACAAAAGTCACGCC – 3’

Amplification of *fimH* flanking regions in NRG857c and LF82
AIECflankF1 5’ – GATCTCTAGACGGCTATGTCAGAGATAATGGCT – 3’
AIECflankR1 5’ – *CAAACAGGGTAATAACTCGTTTCAT*TACAATCATCTCTTCGGGTTCAG – 3’
AIECflankF2 5’ – *TGGCGTGACTTTTGTTTATCAATAA*AGAAATCACAGGGCATTGCTAAT – 3’
AIECflankR2 5’ – GATCGAGCTCATCCACTGCTCATAGTTGCCA – 3’

Primers F1 and R1 amplify a 977 base pair region directly upstream of *fimH*
Primers F2 and R2 amplify a 973 base pair region directly downstream of *fimH*
Underlined base pairs are selected restriction enzyme sites
Italicized base pairs are complementary regions to *fimH*

Amplification of *fimH* flanking regions in K-12
K12flankF1 5’ – GATCTCTAGAGCTATGTCAGGGATAACGGCT – 3’
K12flankR15’ – *CAAACAGGGTAATAACTCGTTTCAT*TACAATCATCTCTTCGGGTTCAG – 3’
K12flankF2 5’ – *TGGCGTGACTTTTGTTTATCAATAA*AGAAATCACAGGACATTGCTAAT – 3’
K12flankR2 5’ – GATCGAGCTCGCCATCATTCCTGAAAGCA – 3’

Primers F1 and R1 amplify a 975 base pair region directly upstream of *fimH*
Primers F2 and R2 amplify a 966 base pair region directly downstream of *fimH*
Underlined base pairs are selected restriction enzyme sites
Italicized base pairs are complementary regions to *fimH*

Methods

Construction of suicide vector pRE112-Gen

The gentamicin resistance cassette was PCR amplified from pUCGm (NCBI GI: 440199) using the primers pUCGmF-2 and pUCGmR and PCR cycling conditions: 94°C 1 minute, 52°C 1 minute, 68°C 1 minute. The PCR product and the suicide vector pRE112 were digested with ApaI (New England Biolabs, Canada), ligated together and transformed into chemically competent DH5α λ pir cells. Successful clones were selected on gentamicin (15 µg/mL).

Construction of FimH-FimC-His and FimC-His expression vectors

To clone into the multiple cloning site-1 of the expression vector pCOLADuet-1 (162), *fimH* was PCR amplified from NRG857c, LF82 and K-12 genomic DNA using the primers fimHpurFNcoI and PCR cycling conditions: 94°C 1 minute, 53°C 1 minute, 68°C 1 minute. The PCR product and the cloning vector pCOLADuet-1 were digested with NcoI (New England Biolabs, Canada) and SalI (New England Biolabs, Canada), ligated together and transformed into chemically competent DH5α cells. Clones were selected on kanamycin (50 µg/mL) and sequenced using the primers DuetUP1 and DuetDOWN1.

To clone *fimC*-His into the multiple cloning site-2, *fimC* was PCR amplified from genomic DNA using fimCpurF and fimCHispurR and PCR cycling conditions: 94°C 1 minute, 55°C 1 minute, 68°C 1 minute. To clone truncated *fimC*-His lacking the

periplasmic signal sequence, the primers fimCpurfwdGGA and fimCHispurR and the same PCR cycling conditions were used. The PCR product and pCOLADuet-1 were digested with BglII (New England Biolabs, Canada) and XhoI (New England Biolabs, Canada), ligated together and transformed into chemically competent DH5α cells. Clones were selected on kanamycin (50 µg/mL) and sequenced using the primers DuetUP2 and DuetDOWN2.

Sequencing *fimH* in CDEC isolates BKC 28-1, 28-3, 28-21 and 28-42

fimH was PCR amplified from genomic DNA using the primers AIECfimHscrfwd and AIECfimHscrrev and PCR cycling conditions: 94°C 5 minutes, [94°C 1 minute, 51°C 1 minute, 68°C 1 minute] x 35 cycles. PCR products were purified using the Qiaquick PCR Purification kit and sequenced using the same primers. Sequences were analyzed using ClustalW2.

Western blot analysis of FimC-His expression

pNRGFimC-His and pK12FimC-His plasmids were transformed into BL21-DE3 chemically competent cells. Clones were selected on kanamycin (50 µg/mL). Strains were grown overnight in 3mL LB + kanamycin, and subcultured 1:100 into 5 mL LB + kanamycin and grown for 3.5 hours. Cells were induced with 1mM isopropyl β-D-1-thiogalactopyranoside for 3 hours. Both uninduced and induced samples were collected, spun down and resuspended in 20 µL of 5X sample buffer. 5 µL of sample was separated on a 12% SDS-PAGE gel. The gel was either stained with Coomassie Blue R-250 or

transferred to a polyvinylidene fluoride membrane which was blocked for one hour with 5% skim milk solution and probed with 1:2500 mouse anti-His antibody (Sigma) overnight at 4°C. The membrane was washed three times with Tris-buffered saline, 0.1% Tween-20 (TBST), incubated with 1:5000 anti-mouse secondary antibody for 1 hour, washed three times with TBST and developed using a 30 second exposure time.

Purification of FimHC-His complex (protocol developed and purification performed by Kun Zhang)

pNRGFimHC-His was transformed into BL21-DE3 chemically competent cells. Clones were selected on kanamycin (50 µg/mL). Several colonies were inoculated into 10mL LB + kanamycin and grown overnight. Bacteria were sub-cultured 1:100 into 2L of LB media containing kanamycin (50 µg/mL). Bacteria were incubated at 37°C with aeration until OD_{600} ~ 0.6. 1mM isopropyl β-D-1-thiogalactopyranoside was added and bacteria were grown for 3 hours at 37°C with aeration. Cells were harvested and resuspended in NiA buffer (40mM Tris, pH 7.5, 500mM NaCl). Cells were sonicated three times (energy transfer ~3000J). A protease inhibitor cocktail containing phenylmethylsulfonyl fluoride, pepstatin A, leupeptin and benzamidine was added before and after sonication to prevent proteolytic degradation. Cells were spun down (9000 rpm, 1hr, 4°C, FX6100 rotor). Supernatant was collected and spun down (13600 rpm, 12 minutes). Supernatant was collected and incubated with leupeptin, benzamidine and DNAse at room temperature for 3 min. Supernatant was filtered using 0.2 micron filter.

Ni-Nitrilotriacetic acid beads were equilibrated with 50mL NiA buffer. Filtered supernatant was added onto the column. The column was washed with 100mL 10mM imidazole, 50mL 20mM imidazole and 25mL 40mM imidazole. Protein was eluted with 3x5mL 80mM imidazole and 3x5mL 320mM imidazole.

45mL SA buffer (20mM Tris, pH 7.0) was added to 5mL elution fractions containing FimHC. S column was equilibrated with 10% SB (20mM Tris, pH 7.0, 0.5M KCl). Protein was filtered using 0.2 micron filter and added to column. Column was successively washed with 20% SB, 40% SB, 80% SB and 100% SB.

Protein was concentrated using a 10K concentrator and stored in 20% glycerol, 20mM Tris, 150mM KCl.

Results

Mutation analysis of the *fimH* sequence in four representative CDEC isolates

To determine the presence of *fimH* mutations in AIEC isolates from the CDEC collection, *fimH* was sequenced in four representative strains of unique serotype isolated from three CD patients. We determined that two strains, BKC28-3 and BKC28-42 had an identical FimH primary sequence to NRG857c FimH. BKC28-1 possessed three mutations Ala25Thr, Ser70Asn and Asn78Ser and BKC28-21 possessed two mutations Ser70Asn and Asn78Ser. Thus, there is some diversity in the FimH sequence of AIEC isolates.

Strain CDEC designation	Strain	Serotype (determined by PHAC)	Missense mutation relative to NRG857c mature FimH sequence [NRGaa] aa# [CDECaa]
1-1-1	BKC 28-1	OR:H4	Ala25Thr, Ser70Asn, Asn78Ser
1-1-3	BKC 28-3	O6:NM	No change
2-1-1	BKC 28-21	O166:H15	Ser70Asn, Asn78Ser
3-1-2	BKC 28-42	OR:H31	No change

Table 1. Mutation analysis in FimH sequence of selected CDEC isolates.

NRG857c FimC-His and K-12 FimC-His expression is inducible.

To confirm that FimC-His expression is inducible using the pCOLA-Duet vector, pNRGFimC-His and pK12FimC-His vectors were expressed in *E. coli* BL21-DE3. Protein expression was induced with IPTG and accessed via Western blot analysis using an anti-His antibody (Sigma). We did not observe any protein expression in the uninduced fraction indicating tight control of expression. After induction, we observed two bands at ~23 kDa corresponding to full-length FimC-His and 21 kDa corresponding to truncated FimC-His lacking the periplasmic signal sequence.

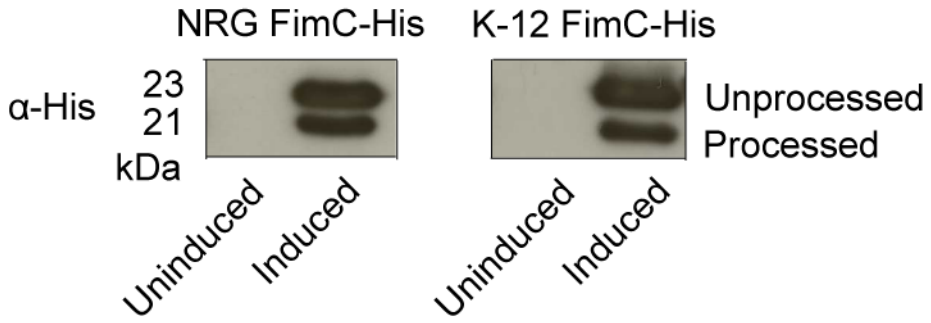

Figure 10. NRG857c FimC-His and K-12 FimC-His is inducible using a pCOLADuet-1 vector. IPTG-inducible expression of FimC encoding a C-terminal His tag was assessed by Western blot analysis. Uninduced and induced samples were probed with mouse anti-His antibody (1:2500, Sigma). Two bands at approximately 23 and 21 kDa were observed corresponding to cytoplasmic FimC (+ signal sequence) and periplasmic FimC (- signal sequence).

NRG857c FimH-FimC-His complex purification via Ni-NTA chromatography and cation exchange chromatography.

NRG857c FimH-FimC-His was expressed in *E. coli* BL21-DE3 following IPTG induction. Using a Ni-NTA column followed by an S column, the FimH-FimC-His complex was purified with few remaining impurities.

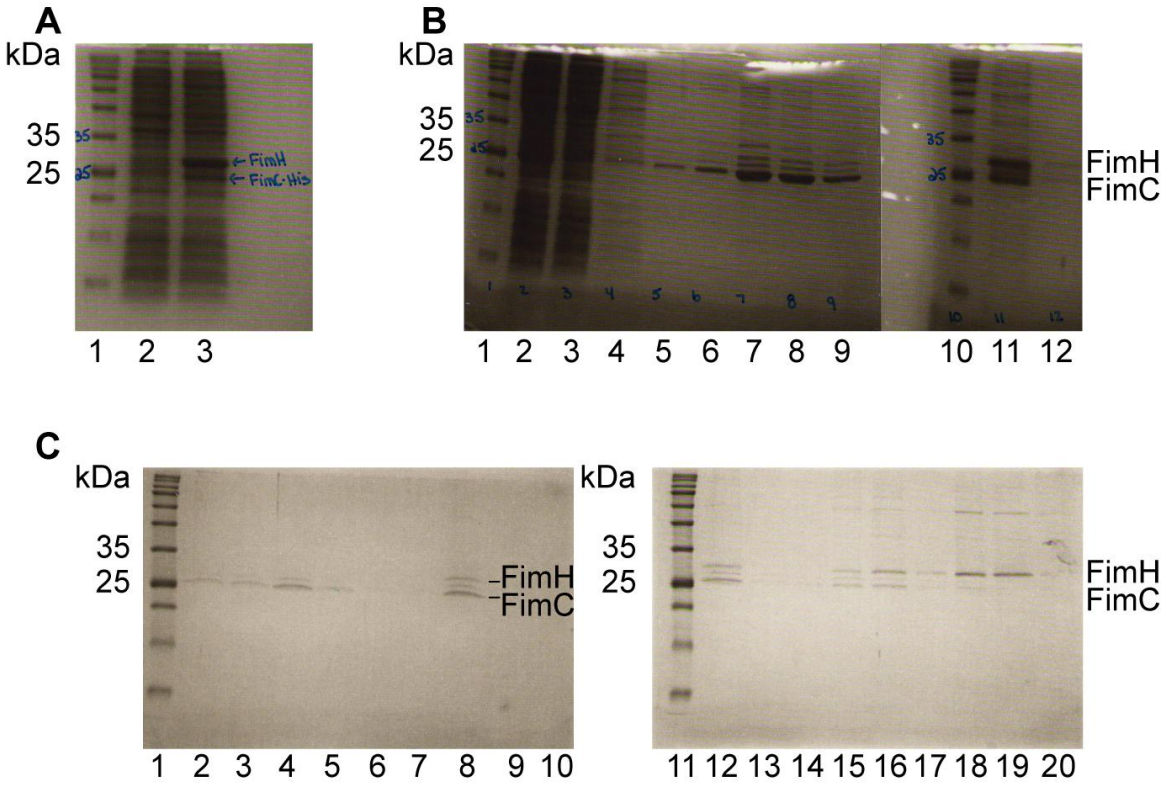

Figure 11. NRG857c FimH-FimC-His induction and purification. FimH (~30 kDa) and FimC-His (~23 kDa) were expressed in BL21-DE3 after IPTG induction. Lane 1 – Marker; Lane 2 – Uninduced sample; Lane 3 – Induced sample (A). Ni-NTA purification was initially used to purify NRG857c FimH-FimC-His. Lane 1 – Marker; Lane 2 – Lysate sample; Lane 3 – Flowthrough (FT); Lane 4 – 10mM imidazole FT; Lane 5 – 20mM imidazole FT; Lane 6 – 40mM imidazole FT; Lane 7 – 80mM imidazole elute 1; Lane 8 – 80mM imidazole elute 2; Lane 9 – 80mM imidazole elute 3; Lane 10 – 320mM imidazole elute 1; Lane 11 – 320mM imidazole elute 2 (B). The collected elute was then purified on an S column. Lane 1 – Marker; Lane 2-Lane 7 – 20% SB fractions; Lane 8 – Sample; Lane 9-10 – Blank; Lane 11 – Marker; Lane 12 – Sample; Lane 13 – FT; Lane 14-16 – 40% SB fractions; Lane 17-20 – 80% SB fractions.