ORIGINAL ARTICLE

Colonic mucosa-associated diffusely adherent *afaC+ Escherichia coli* expressing *lpfA* and *pks* are increased in inflammatory bowel disease and colon cancer

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ABSTRACT

Objective Colonic mucosa-associated *Escherichia coli* are increased in Crohn's disease (CD) and colorectal cancer (CRC). They variously haemagglutinate, invade epithelial cell lines, replicate within macrophages, translocate across M (microfold) cells and damage DNA. We investigated genes responsible for these effects and their co-association in colonic mucosal isolates.

Design A fosmid library yielding 968 clones was prepared in *E coli* EPI300-T1 using DNA from a haemagglutinating CRC isolate, and resulting haemagglutinating clones were 454-pyrosequenced. PCR screening was performed on 281 colonic *E coli* isolates from inflammatory bowel disease (IBD) (35 patients), CRC (21) and controls (24; sporadic polyps or irritable bowel syndrome).

Results 454-Pyrosequencing of fosmids from the haemagglutinating clones (n=8) identified the afimbrial adhesin *afa-1* operon. Transfection of *afa-1* into *E coli* K-12 predictably conferred diffuse adherence plus invasion of HEp-2 and I-407 epithelial cells, and upregulation of vascular endothelial growth factor. *E coli* expressing *afaC* were common in CRC (14/21, p=0.0009) and CD (9/14, p=0.005) but not ulcerative colitis (UC; 8/21) compared with controls (4/24). *E coli* expressing both *afaC* and *lpfA* (relevant to M-cell translocation) were common in CD (8/14, p=0.0019) and CRC (14/21, p=0.0001), but not UC (6/21) compared with controls (2/24). *E coli* expressing both *afaC* and *pks* (genotoxic) were common in CRC (11/21, p=0.0015) and UC (8/21, p=0.022), but not CD (4/14) compared with controls (2/24). All isolates expressed *dsbA* and *htrA* relevant to intra-macrophage replication, and 242/281 expressed *fimH* encoding type-1 fimbrial adhesin.

Conclusions IBD and CRC commonly have colonic mucosal *E coli* that express genes that confer properties relevant to pathogenesis including M-cell translocation, angiogenesis and genotoxicity.

Significance of this study

What is already known about on this subject?

- Mucosa-associated *E coli* are increased in CD and colon cancer.
- These *E coli* adhere to and invade intestinal epithelial cells in culture, and replicate within macrophages.
- These properties have led to them being designated AIEC. However, no genotype is consistent across all AIEC isolates.

What are the new findings?

- There is an increased prevalence of *afaC+* DAEC isolates associated with the colonic mucosa in CD and colon cancer
- The presence of the afimbrial adhesin operon correlates with diffuse adherence to, invasion of, and increased *VEGF* expression in intestinal epithelial cells
- Colonic mucosal *E coli* that are diffusely adherent and that bear key genes potentially relevant to CD (conferring mucosal invasion and translocation and replication in macrophages), as well as to colon cancer pathogenesis (conferring the ability to induce angiogenesis and genotoxicity) are strongly associated with colon cancer and IBD, and could be relevant to pathogenesis.

How might it impact on clinical practice in the foreseeable future?

- Interventions that either reduce colonisation by DAEC or block their interaction with the mucosa may have preventive or therapeutic effects in colon cancer and CD.

INTRODUCTION

It is accepted that bacteria are involved in inflammatory bowel disease (IBD) pathogenesis but the mechanisms are poorly understood.[1] An increase in mucosa-associated *Escherichia coli* in Crohn's disease (CD) has been found in both ileum[2 3] and colon.[4–7] Increased mucosa-associated *E coli* have also been reported in colorectal cancer (CRC)[5 8] and to a lesser extent in ulcerative colitis (UC).[9–11] These *E coli* typically adhere to and invade intestinal epithelial cells in culture and replicate within macrophages.[12 13] Gentamicin treatment of CD intestinal biopsies followed by lysis and culture

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implies the presence of intracellular *E coli*,[5 8 14] and *E coli* DNA has been demonstrated within a majority of CD granulomas.[15]

Designated as 'adherent, invasive *E coli*' (AIEC), some of the properties of these *E coli* have been associated with specific genes, particularly in studies of the 'paradigm' ileal AIEC, LF82.[16] These include high-temperature requirement-A (*htrA*) and oxidoreductase disulfide bond-A protein (*dsbA*), which support intra-macrophage survival[17 18] and long polar fimbriae (*lpfA*) involved in translocation across M cells of the follicle-associated epithelium (FAE).[19] There is, however, no genotype that is consistent across all AIEC. Moreover, their invasion of epithelial cell lines *in vitro* varies between cell lines and is a property found in other *E coli* including diffusely adherent *E coli* (DAEC) and uropathogenic *E coli* (UPEC).[20 21] We have previously shown that colonic mucosa-associated *E coli* from CD and CRC commonly expressed haemagglutinins and that this correlated with their ability to adhere to and invade epithelial cell lines.[5]

Recent studies show that *E coli* possessing the polyketide synthase gene complex (*pks*) responsible for producing the genotoxin colibactin induce inflammation-associated CRC in mice[22] and are commonly mucosa associated in sporadic CRC.[22 23]

Here we have used a fosmid-clone library, based on genomic DNA derived from a colon cancer mucosal AIEC, to investigate the nature of the haemagglutinin gene(s), its relevance to the AIEC phenotype, and distribution among mucosal *E coli* from IBD, CRC and controls. We also screened these isolates for *htrA* and *dsbA*, relevant to replication within macrophages, *lpfA* and *fimH* relevant to M-cell translocation, a likely portal of entry for mucosal invasion in CD and for the *pks* gene complex, relevant to CRC pathogenesis.

METHODS

Cell culture

The human colon adenocarcinoma cell-line Caco2 (#86010202), Burkitt's lymphoma cell-line Raji-B (#85011429) and J774A.1 murine macrophages (#91051511) were from ECACC (Wiltshire, UK). Human I-407 (CCL-6) and HEp-2 (CCL-23) cells were from the American Type Culture Collection (LGC Standard; Teddington, UK). Caco2-cl1 cells, kindly provided by Dr Elisabet Gullberg (Linköping University, Sweden), were originally obtained from Dr Maria Rescigno (European Institute of Oncology; Milan, Italy).[24] Caco2, Caco2-cl1 and I-407 were cultured in DMEM, HEp-2 in MEM, Raji-B and J774A.1 in RPMI-1640; supplemented with 10% FBS, 4 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and maintained at 37°C in 5% carbon dioxide.

Generation of an *E coli* library from a colonic mucosa-associated haemagglutinating *E coli* with the AIEC phenotype

Using *E coli* HM358, a haemagglutination-positive isolate from a CRC patient[5] as the parent strain, a randomly sheared, non-biased fosmid library was constructed (CopyControl fosmid library kit; Epicentre, Wisconsin, USA). Genomic DNA extracted from HM358 (Genomix Cell/Tissue kit: Talent; Italy) was sheared, end-repaired with 5′-phosphorylated blunt ends, 30–50 kb DNA fragments ligated to phosphatase-treated fosmid pCC1FOS and introduced into phage T1-resistant *E coli* K-12 derivate EPI300-T1. *E coli* clones were selected on Luria–Bertani chloramphenicol (12.5 µg/mL) agar.

Haemagglutination assay

E coli clones were screened for haemagglutination of 1% group O human erythrocytes (NBS; Liverpool, UK)[5] with haemagglutination-positive *E coli* HM358 and haemagglutination-negative *E coli* EPI300-T1/pCC1FOS as controls.

454-Pyrosequencing

Following the induction of high copy number of fosmids during bacterial growth (Epicentre copy-control auto-induction solution), clones from haemagglutination-positive *E coli* were extracted using Qiafilter midi-kits (Qiagen; Crawley, UK). 454-Sequencing was performed using the GS-FLX Titanium Series (Roche-454 Life Science; Branford, USA). 454-Reads were assembled with $Newbler_{v2.0}$ and protein-coding sequences identified with GeneMark-$P_{v2.4}$ (http://exon.biology.gatech.edu/). Predicted open-reading frames and translated amino acids were subjected to BLASTP to identify homologies to operons. Gene representation was performed using $Artemis_{v13}$ (http://www.sanger.ac.uk/resources/software/artemis/).

Following sequence analysis, *E coli* HM358 and all fosmid-containing haemagglutination-positive *E coli* were screened by PCR for afimbrial adhesin (*afaC*), P-fimbriae (*papC*), type-1 pili (*fimA*/*fimC1*/*fimH*), outer-membrane protein-C (*ompC*) and flagellin (*fliC*). Primers and conditions are detailed in supplementary file S1 (available online only).

Presence of *afa* operon in colonic mucosal *E coli* strains

A panel of 281 colonic mucosal *E coli* previously isolated from 80 patients was studied;[5] 71 from 14 CD patients, 51 from 21 UC patients, 120 from 21 CRC patients and 39 from 24 controls. IBD and control isolates were obtained from colonoscopic biopsy of non-ulcerated sigmoid colon, CRC isolates from biopsy of resection specimens.[5] PCR to detect all *afa* strains, irrespective of *afaE* subtype, was performed using primers to amplify *afaC*, present in all operons of the afimbrial adhesin family (*afa-1*,*-2*,*-3*,*-5*,*-7*,*-8* and *daa*).[25] DAEC C1845, provided by Professor Alain Servin (INSERM-UMR756, Châtenay-Malabry, France), which carries the *daa* operon[26] and CD AIEC LF82 lacking *afa*,[16] were used as controls. Additional ileal CD isolates LF10, LF11, LF86 and LF13, provided by Professor Arlette Darfeuille-Michaud (INSERM-UMR1071, Clermont-Ferrand, France),[2] were also screened for the *afa* operon.

Generation of an *afa-1*-expressing recombinant *E coli*

Following sequence identification of Afa-1 as the putative adhesin responsible for haemagglutination, purified fosmid was extracted from haemagglutination-positive clone *E coli* 8H8. The complete *afa-1* operon (6.8 kb) was isolated by *Xba*I/*Spe*I restriction enzyme digest, purified by gel electrophoresis, ligated into pUC18 using T4 DNA-ligase and propagated in *E coli* One-ShotTOP10 (Invitrogen) selected on 100 µg/mL ampicillin agar. pUCAfa was then used to transform chemically competent *E coli* EPI300-T1 containing empty fosmid, to allow direct comparison with *E coli* 8H8. The presence and orientation of *afa-1* within pUCAfa were confirmed by PCR. Functional adhesin was confirmed by haemagglutination and adhesion to HEp-2 cells (see supplementary file S2, available online only).

Diffuse adherence to HEp-2 cells

The ability of *E coli* isolates, fosmid and pUCAfa-transformed constructs to mediate diffuse adherence to HEp-2 was assessed in the presence of 1% methyl-α-D-mannopyranoside to exclude

type-1 fimbriae-mediated adhesion,[27] with DAEC C1845 included as positive control.[26]

Adherence and invasion to intestinal epithelial cell lines

Adherence to, and invasion of, *E coli* to HEp-2, I-407 and differentiated Caco2 cells (15d post-confluent) was assessed by gentamicin protection assay in the presence of methyl-α-D-mannopyranoside.[5] Bacteria were cultured overnight on Luria–Bertani agar, with adherence and invasion calculated as the percentage of the original inoculum, and data expressed relative to wild-type AIEC HM358.

Real-time PCR for *VEGF*

Confluent I-407 cells (8×10^5 cells/well) were serum-starved for 24 h and then infected for 4 h Multiplicity of infection 20 (MOI 20) with either CRC AIEC HM358, *E coli* EPI300-T1/pCC1FOS transformed with *afa-1* operon (pUCAfa) or vector alone (pUC18), or DAEC C1845 (known to upregulate vascular endothelial growth factor (*VEGF*)).[20] Total RNA was isolated (RNeasy kit; Qiagen) and quantified by NanoDrop. *VEGF* messenger RNA was assessed by quantitative PCR of first-strand synthesised complementary DNA (Roche, Burgess Hill, UK) in a Lightcycler480 system using human *VEGF* or *β-actin* specific primers (Eurogentec) and probes from the Roche Universal Probe Library (see online supplementary file S1, available online only).

Replication of E coli within J774A.1 macrophages

Macrophages were seeded onto 24-well plates (10^5 cells/well) for 24 h and infected (MOI 10) with either *E coli* HM358, library clones or *E coli* constructs in antibiotic-free media. Intra-macrophage replication was determined by recovery of intracellular bacteria from lysed gentamicin-treated cells after 6 h or 24 h, relative to intracellular numbers at 3 h.[13]

Translocation through M cells in culture

Translocation through M cells, generated by co-culture of Caco2-cl1 and Raji-B cells, was conducted as previously described.[28] Successful M-cell generation was confirmed by translocation of CD *E coli* HM605 and 0.5 μm yellow-green FluoSpheres (Invitrogen). Trans-epithelial electrical resistance was monitored throughout.

Screening for intra-macrophage replication genes *htrA* and *dsbA, lpfA* and the genotoxin-producing *pks* pathogenicity island in colonic mucosal *E coli*

PCR assays for genes relevant to AIEC intramacrophage survival and replication, *dsbA* and *htrA*,[17 18] and the two major *lpf* operons ($lpfA_{Shigella}$ and $lpfA_{LF82}$) identified in ileal CD AIEC,[19] were performed. PCR for *pks* prevalent in patients with IBD and CRC, was performed previously.[22] Primers detailed in supplementary file S1 (available online only).

Statistics.

N is the total number of independent experiments performed, with n replicates for each treatment group. Independent groups were assessed for normality and equality of variance, and analysed using Mann–Whitney U or Kruskal–Wallis followed by pair-wise comparison of treatment means (StatsDirect$_{v2.6.2}$; Sale, UK). Comparing PCR datasets, Fisher's exact and χ^2 tests were utilised as appropriate, in which N is the number of patients, and n is *E coli*. Differences were considered significant at $p<0.05$.

RESULTS

Fosmids extracted from haemagglutinin-positive *E coli* in the clone library derived from CRC mucosal *E coli* HM358 share a common region containing the afimbrial adhesin *afa-1* operon.

A total of 968 fosmid clones was generated from randomly sheared 30–50 kb DNA fragments of *E coli* HM358, a haemagglutination-positive CRC colonic mucosal isolate. Eight of the resulting 968 *E coli* library clones were strongly haemagglutination positive. Analysis of 454-sequence data identified

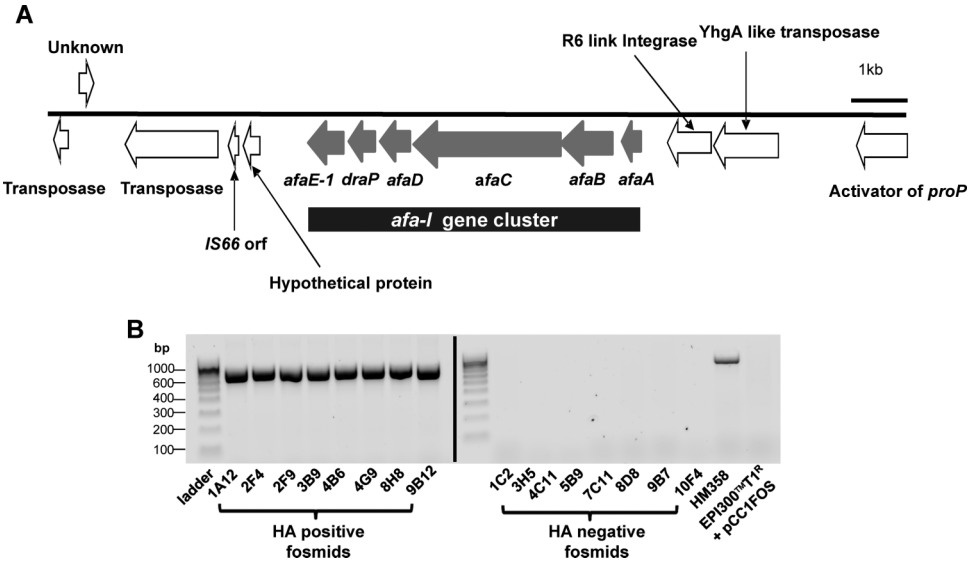

Figure 1 Haemagglutinin-positive *E coli* in the fosmid clone library derived from *E coli* HM358 share a common region containing the afimbrial adhesin *afa-1* operon. (A) Genomic organisation of the common region shared by haemagglutination (HA)-positive clones includes *afaA* (encoding a transcriptional regulator); *afaB* (chaperone); *afaC* (usher); *afaD* (invasin); *draP* (linker element) and *afaE-1* (an adhesin). (B) PCR for *afaC*. A 672 bp fragment is detected in all eight haemagglutination-positive clones and *E coli* HM358. *E coli* EPI300-T1/pCC1FOS and eight haemagglutination-negative fosmids lacked *afaC*.

only the full *afa-1* operon, coding for afimbrial adhesin Afa-1, as common to the haemagglutination-positive fosmids, plus some additional surrounding transposase and integrase elements (figure 1A; see supplementary file S3, available online only). The *afa* operon identified contained six open-reading frames encoding for AfaA (a transcriptional regulator), AfaB (a periplasmic chaperone), AfaC (an usher), AfaD (an invasin), DraP (a linker element found in the *dra* operon) and AfaE-1 (the mannose-resistant adhesin); figure 1A. PCR targeting a 672 bp amplicon of *afaC* confirmed the *afa* operon within the parent *E coli* HM358 and all eight haemagglutination-positive fosmid clones, whereas eight haemagglutination-negative clones chosen at random from the library and *E coli* EPI300-T1/pCC1FOS were *afa* negative (figure 1B). The nucleotide sequence of the HM358 *afa-1* cluster was deposited in GenBank; accession no. JN688153.

PCR identified that all eight fosmid-containing haemagglutination-positive *E coli* were positive for *fliC*, *fimA*, *fimC1*, *fimH* and *ompC* but negative for *papC* and *lpfA*.

Increased prevalence of *afa in* mucosal *E coli* isolates from CD and CRC patients

PCR for *afaC* (present in all operons of the afimbrial adhesin family) in a large panel of colonic mucosal *E coli* isolates showed increased prevalence of *afa* among isolates from CD (nine of 14 patients; p=0.005, Fisher's exact test) and CRC (14 of 21; p=0.0009), but not UC (eight of 21), compared with controls (four of 24); table 1). When expressed using *E coli* as denominator, the presence of *afaC* was also more common among isolates from CD (39 of 71 isolates (54%)), UC (28/51 (54%)) and CRC (73/120 (60%)) compared with controls (11/39 (28%)); all p≤0.02, with CRC versus controls p=0.0008, χ^2; table 2. Like AIEC LF82, ileal CD isolates LF11 and LF86 were negative for *afaC*, whereas ileal isolates LF10 and LF13 were positive.

Presence of the *afa-1* operon correlates with diffuse adherence to and invasion of HEp-2 cells

After 6 h infection of HEp-2 cells, AIEC HM358 and all eight haemagglutination-positive (*afa-1* possessing) library clones demonstrated diffuse adherence to HEp-2 characteristic of DAEC (figure 2A–C). No adherence to HEp-2 was observed with eight randomly-selected haemagglutination-negative clones, nor with the K-12 plating strain *E coli* EPI300-T1/pCC1FOS. All the haemagglutination-positive (*afa-1* possessing) *E coli* invaded HEp-2 cells, at levels similar to those observed for *E coli* HM358. The haemagglutination-negative (*afa-1* negative) clones, *afa*-negative AIEC LF82, and the K-12-derived EPI300-T1 plating strain, were all substantially less invasive to HEp-2; p<0.0001; Kruskal–Wallis (figure 2D).

Table 1 Prevalence of *afaC* positive *E coli* in patients with CD, colitis and colon cancer compared with controls (using total number of patients as the denominator)†

	Total no. of patients	*afaC*+	p Value*
CD	14	9	0.005
CRC	21	14	0.0009
UC	21	8	NS
Controls	24	4	

*p Values obtained using Fishers exact test (2P component).
†Presence or absence of *afaC* is based on PCR assay.
afaC, gene encoding afimbrial adhesin outer membrane usher protein; CD, Crohn's disease; CRC, colorectal cancer; UC, ulcerative colitis.

Table 2 Presence of *afaC* in *E coli* isolated from patients with CD, colitis and colon cancer compared with controls (using total number of *E coli* as the denominator)†

	afaC+	*afaC*–	p Value*
CD	39	32	0.0127
CRC	73	47	0.0008
UC	28	23	0.0204
Controls	11	28	

*p Values obtained using χ^2 test (Yates-corrected).
†Presence or absence of *afaC* is based on PCR assay.
afaC, gene encoding afimbrial adhesin outer membrane usher protein; CD, Crohn's disease; CRC, colorectal cancer; UC, ulcerative colitis.

Presence of the *afa-1* operon correlates with the ability of *E coli* to adhere to and invade intestinal epithelial cells

The eight haemagglutination-positive *E coli* clones possessing *afa-1* were all shown to adhere to undifferentiated I-407 cells (adhesion relative to HM358 was 0.55±0.13) (mean±SEM) in contrast to non-haemagglutinating, *afa*-negative *E coli*, 0.12 ±0.02; n=4 p<0.0001, Kruskal–Wallis (figure 3A). Similarly, haemagglutination-positive, *afa-1*-possessing *E coli* invaded I-407 cells (invasion relative to HM358 was 0.31±0.13) in contrast to haemagglutination-negative, *afa*-negative clones (0.01 ±0.01); p<0.0001 (figure 3B). Haemagglutination-positive *E coli* clones possessing *afa-1* also demonstrated greater adhesion to fully differentiated Caco2 cells (*afa*-positive clones, 0.74 ±0.14 compared to *afa*-negative clones, 0.3±0.02); n=4, p<0.0001 (figure 3C). Of note though, three of eight haemagglutination-negative (*afa-1* negative) *E coli* were able to invade differentiated Caco2 (figure 3D). The plating strain EPI300-T1/pCC1FOS showed negligible adhesion to and invasion of both cell lines.

Transfection of the full *afa-1* operon (pUCAfa) into the plating strain resulted in increased adhesion to and invasion of I-407 cells compared to the plating strain transformed with pUC18; p<0.0001, Kruskal–Wallis (figure 3E,F). Similar results were obtained using differentiated Caco2 cells (data not shown).

Assessment of the *afa*C status of 24 colonic mucosal isolates previously assessed for adhesion to and invasion into I-407 epithelial cells[5] showed that nine of 13 *afa*C-positive isolates were invasive to I-407 cells. However, various *afa*C-negative isolates, including LF82, were also observed to be invasive for this cell line.

Presence of *afa-1* in *E coli* upregulates *VEGF* expression by intestinal epithelial cells

VEGF mRNA was upregulated 3.07±0.16-fold in I-407 cells infected with *E coli* EPI300-T1/pCC1FOS transformed with the *afa-1* operon (pUCAfa) and 3.71±0.22-fold with AIEC HM358, compared to uninfected cells (N=4, n=3; p<0.001 Kruskal–Wallis). The level of response was similar in cells infected with DAEC C1845 (N=2, n=3; p<0.001); figure 4.

The presence of Afa-1 in mucosally associated *E coli* does not confer ability to replicate within macrophages or translocate through M cells

In contrast to the parent isolate HM358, no significant intramacrophage replication was seen with any of the eight haemagglutination-positive (*afa-1* possessing) *E coli* clones (see

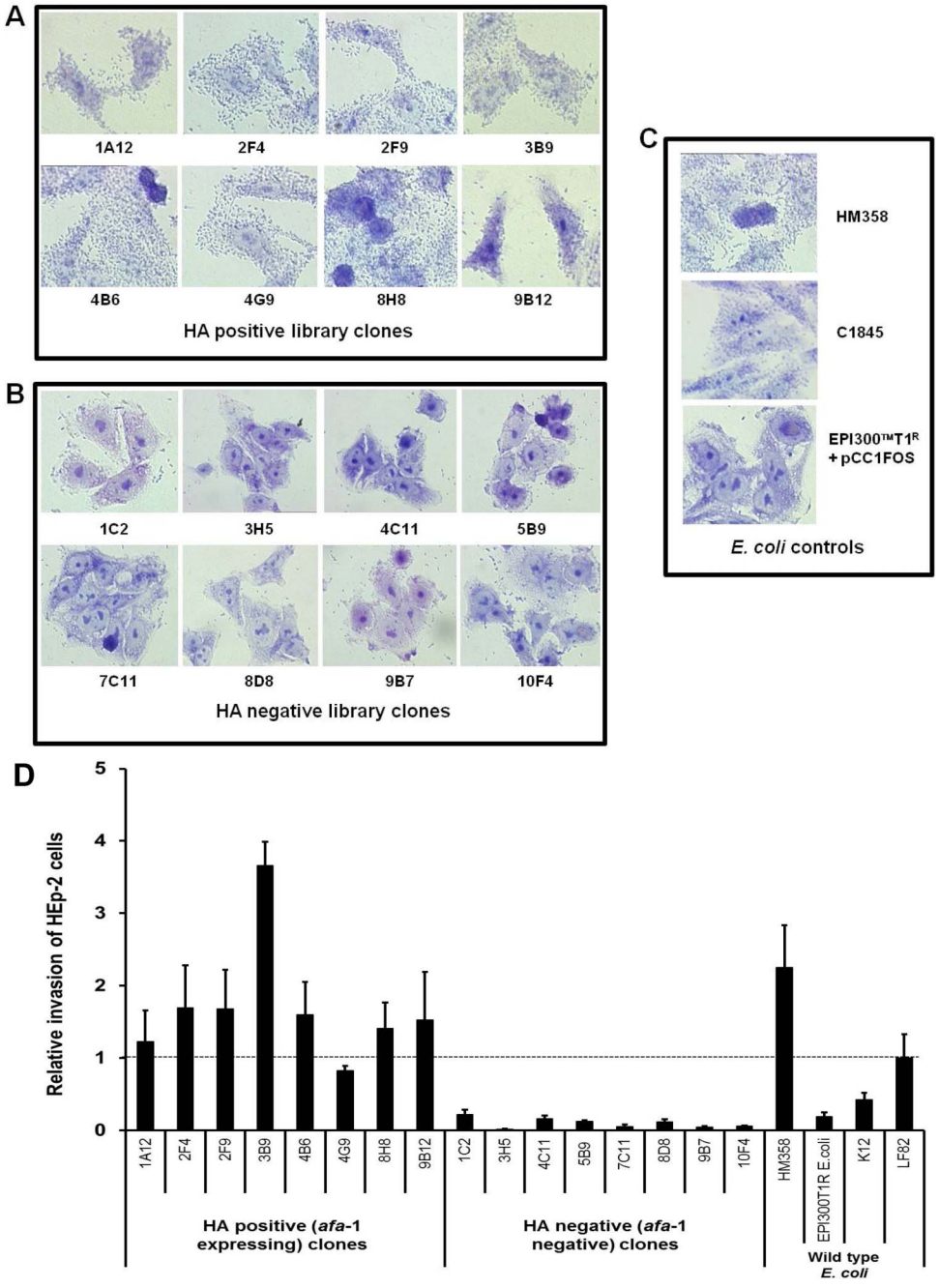

Figure 2 The presence of the *afa-1* operon in haemagglutinating *E coli* correlates with diffuse adherence and invasion to HEp-2 epithelial cells. Giemsa stain of HEp-2 cells infected with *E coli* strains. (A) All eight haemagglutinin-positive library clones showed diffuse adherence to cell cultures. (B) Eight haemagglutination (HA)-negative fosmid clones chosen at random from the library were non-adherent. (C) Colonic mucosally associated *E coli* HM358 exhibited a diffusely adherent pattern as per diffusely adherent *E coli* C1845. The *E coli* K12 plating strain EPI300T1 containing pCC1Fos was non-adherent. (D) The eight haemagglutination-positive fosmid library clones possessing the *afa-1* gene cluster, exhibiting diffuse adherence, showed increased ability to invade Hep-2 cells compared to haemagglutination-negative clones. Invasion calculated as percentage of the original inoculums (multiplicity of infection 10) and expressed relative to *E coli* LF82 previously shown to be invasive in this cell line.[2] * $p<0.05$ and *** $p<0.001$ when compared to the non-invasive plating strain EPI300-T1 containing pCC1Fos alone (mean±SEM; N=3 experiments, each performed with n=3 replicates; Kruskal–Wallis).

supplementary file S4, available online only). Likewise, seven of the eight haemagglutination-negative clones tested (excepting 4C11), and the *E coli* EP1300-T1 plating strain containing pUCAfa, were unable to replicate within macrophages. Moreover, following initial infection and gentamicin treatment, fewer recombinant *E coli* containing *afa-1* were internalised within macrophages ($0.6\pm0.1\times10^4$ CFU/well) compared with *E coli* containing pUC18 alone ($11.0\pm2.1\times10^4$ CFU/well; $p<0.0001$), suggesting that possession of Afa-1 inhibits phagocytosis (table 3). It was also observed that haemagglutination-positive fosmid library clones possessing the *afa-1* operon, showed no significant increased ability above haemagglutination-negative (*afa-1* negative) clones to release tumour necrosis factor alpha (TNFα) from macrophages (see supplementary file

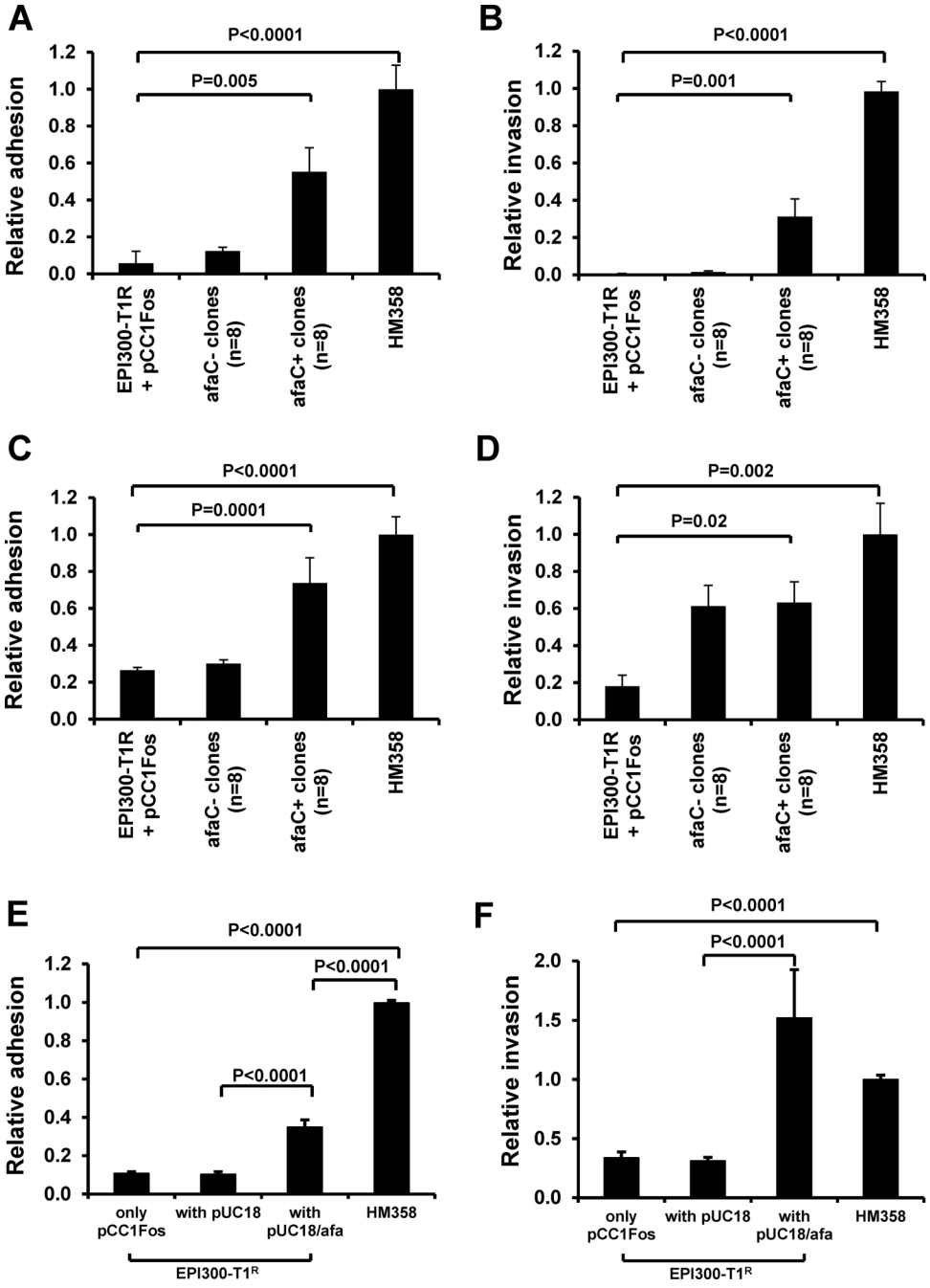

Figure 3 The presence of *afa-1* in haemagglutinating *E coli* correlates with ability to adhere to and invade intestinal epithelial cells. Relative ability of eight haemagglutination-positive library clones possessing *afa-1* (*afaC*+ as determined by PCR) and haemagglutination-negative clones (n=8) to (A) adhere to and (B) invade I-407 cells. Data mean (±SEM) relative to that observed by *E coli* HM358; n=4. Increased (C) adherence to, and (D) invasion of differentiated Caco2 cells by *afa-1*-possessing clones was observed. (E and F) *E coli* EPI300-T1/pCC1FOS transformed with *afa-1* (pUCAfa) adheres to and invades I-407 cells. Data expressed relative to plating strain; N=3, each n=3–5 replicates). p Values determined by Kruskal–Wallis. *Afa*, afimbrial adhesin; *afa-1*, afimbrial adhesin operon 1; *afaC*, gene encoding afimbrial adhesin outer membrane usher protein.

S4, available online only). Similar TNFα levels were released from macrophages by AIEC HM358 (1712±236 pg TNFα/mL) and *E coli* K-12 strain EPI300-T1/pCC1FOS expressing Afa-1 from pUCAfa (1742±31 pg/mL) compared to uninfected controls (16±5 pg/mL; n=3). All 281 colonic mucosal *E coli* isolates (including HM358) expressed *dsbA* and *htrA*, relevant to intramacrophage replication.

While HM358 (which expresses *lpfA*) was observed to translocate across M cells, no significant translocation was seen for *E coli* EPI300-T1 pUCAfa, indicating that possession of Afa-1 adhesin does not support FAE transcytosis (see supplementary file S5, available online only).

Increased prevalence in CD and CRC of DAEC possessing *lpfA* and *fimH* relevant to M cell translocation

Screening of 281 colonic mucosally associated *E coli* demonstrated a striking increased prevalence in CD and CRC of isolates possessing *afa* together with *lpfA* (tables 4–7; see

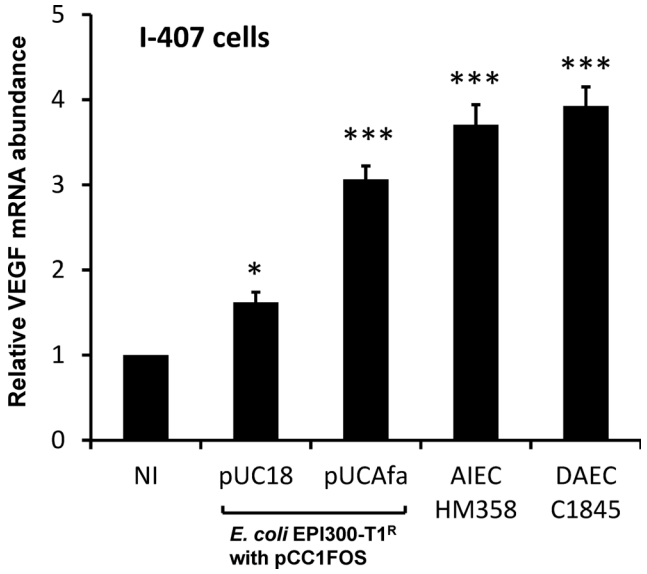

Figure 4 The presence of *afa* in *E coli* upregulates vascular endothelial growth factor (*VEGF*) expression in cultured intestinal epithelial cells. Confluent serum-starved I-407 cells (8×10^5 cells/well) were infected for 4 h with either wild-type colorectal cancer adherent, invasive *E coli* HM358, Afa/Dr diffusely adhering *E coli* C1845 or *E coli* EPI300-T1/pCC1FOS transformed with *afa-1* operon (pUCAfa) or vector alone (pUC18) and total RNA extracted. *VEGF* mRNA measured by quantitative PCR relative to *β actin*. Data mean (±SEM) relative to non-infected cells (set at 100%); N=2–4; each n=3 replicates). *p<0.05, *** p<0.001 determined by Kruskal–Wallis. AIEC, adherent, invasive *E coli*; DAEC, diffusely adherent E coli.

supplementary file S6, available online only). Most isolates (242/281) also expressed *fimH* (61/71 CD, 97/120 CRC, 46/51 UC and 38/39 controls). Only six CD isolates and one cancer isolate expressing both *afaC* and *lpfA* were negative for *fimH*.

Increased prevalence in CRC and UC of DAEC (*afa* positive *E coli*) possessing the *pks* genotoxicity island relevant to carcinogenesis

We have recently shown that colonic mucosal *E coli* from sporadic CRC and IBD commonly express the *pks* genotoxicity island that confers the ability to induce experimental CRC. Screening of *E coli* for co-expression of *afaC* and *pks* shows a marked increase in *afaC+/pks+ E coli* in association with CRC and with UC but not CD (tables 4–7).

Table 3 *E coli* uptake and replication within J774-A1 murine macrophages

Strain	Uptake of bacteria* (CFU/well×10^4)	Fold replication 6 h/3 h†	Fold replication 24 h/3 h†
EPI300-T1			
+ pCC1FOS	9.3±1.6	1.70±0.23	0.69±0.16
+ pCC1FOS/pUC18	11.0±2.1	0.86±0.05	1.21±0.12
+ pCC1FOS/pUC Afa	0.6±0.1‡	1.07±0.06	0.27±0.10
HM358	5.36±1.1	17.66±3.80§	11.26±2.67§

Data expressed as means±SEM, determined from N=2–7 independent experiments, with each experiment performed with n=2–3 replicates.
*CFU recovered from lysed macrophages after 3 h (2 h infection followed by 1 h gentamicin treatment).
†Recovered intracellular bacteria from lysed macrophages after 6 h or 24 h, relative to intracellular numbers at 3 h. Statistical analysis was performed using Mann–Whitney U with Bonferroni correction.
‡Significantly different from EPI300-T1/pCC1FOS+pUC18; p<0.0001.
§Significantly different from EPI300-T1/pCC1FOS; p<0.0001.
CFU, colony-forming units.

Table 4 Prevalence of *afaC+, lpfA+ E coli* in patients with CD, colitis and colon cancer compared with controls (with total patients as the denominator)†

	afaC+, lpfA+	Total no of patients	p Value*
CD	8	14	0.0019
CRC	14	21	0.0001
UC	6	21	NS
Controls	2	24	

*p Values obtained using Fishers exact test (2P component).
†Presence or absence of genes is based on PCR assays.
afaC, gene encoding afimbrial adhesin outer membrane usher protein; CD, Crohn's disease; CRC, colorectal cancer; *lpfA*, gene encoding long polar fimbrial protein; UC, ulcerative colitis.

DISCUSSION

This study shows that colonic mucosa-associated *afa-1*-positive DAEC are increased in CD and CRC. They adhere to and invade intestinal epithelial cells in culture but also commonly express *lpfA* relevant to M-cell translocation that is more likely to be the major initial route for invasion *in vivo*.[19] Colonic mucosal DAEC isolates also possess *htrA* and *dsbA* relevant to survival within macrophages,[17 18] part of the characteristic phenotype of CD-associated AIEC.[12]

Afa-expression confers the ability to induce VEGF expression by epithelial cells, relevant to angiogenesis and tumour development[29 30] and this is confirmed here. We have recently shown that colonic mucosal *E coli* from patients with IBD and CRC more commonly express the *pks* pathogenicity island whose gene products result in the formation of the metabolite colibactin, a genotoxin with the ability to cause epithelial DNA damage and induce tumours in a mouse model of inflammation-associated CRC.[22] Here we show that colonic mucosal *E coli* isolates from CRC and UC commonly express both *pks* and *afaC* together. CD *afaC*-expressing *E coli* isolates, however, did not commonly express *pks*. This may relate to the lack of the increased risk of CRC seen in CD in the absence of colonic involvement, and also raises the possibility that increased *pks* expression by mucosa-associated *E coli* might be a consequence of colitis, because the presence of *pks* has been shown not to affect *E coli*-induced inflammation in the mouse IBD model.[22]

Possession of the *afa-1* operon defines a subgroup of *E coli* that have a characteristic diffuse adherence pattern to HEp-2

Table 5 Presence of *afaC+, lpfA+ E coli* in CD, colitis and colon cancer compared with controls (using total number of *E coli* as the denominator)†

	afaC+, lpfA+	Total no of *E coli*	p Value*
CD	30	71	0.0011
CRC	51	120	0.0005
UC	15	51	NS
Controls	4	39	

*p Values obtained using χ^2 test (Yates-corrected).
†Presence or absence of genes is based on PCR assays.
afaC, gene encoding afimbrial adhesin outer membrane usher protein; CD, Crohn's disease; CRC, colorectal cancer; *lpfA*, gene encoding long polar fimbrial protein; UC, ulcerative colitis.

Table 6 Prevalence of *afaC*+, *pks*+ *E coli* in patients with CD, colitis and colon cancer compared with controls (using total number of patients as the denominator)†

	Total no of patients	*afaC*+, *pks*+	p Value*
CD	14	4	NS
CRC	21	11	0.0015
UC	21	8	0.0222
Controls	24	2	

*p Values obtained using Fisher's exact test (2P component).
†Presence or absence of *afaC* and *pks* is based on PCR assay.
afaC, gene encoding afimbrial adhesin outer membrane usher protein; CRC, colorectal cancer; *pks*, polyketide synthase gene complex; UC, ulcerative colitis.

epithelial cells and that includes UPEC and diarrhoeagenic DAEC strains.[20 21 27 31] Sequencing revealed that the *afa-1* operon identified in our colonic mucosal isolate shares the same linker element *draP* as in the *Dr* operon, which encodes the Afa-related Dr adhesin. Transfection of the *afa-1* operon into a non-pathogenic *E coli* EPI300-T1 (K-12) strain conferred the ability not only to adhere to HEp-2 but also to invade this and other cell lines, thus conferring part of the CD AIEC phenotype. The wild-type strain HM358 shows still greater ability to adhere to and invade than the *afa-1*-transfected *E coli* K-12 strain, implying that other adhesins/invasins are also involved and this is confirmed by invasion to I-407 seen in occasional *afa*-negative isolates.

The *afa-1* operon does not confer the other phenotypic property reported for AIEC and relevant to CD pathogenesis, that is, replication within macrophages. Two genes *htrA* and *dsbA* are already known to support AIEC LF82 replication within macrophages, both encoding stress tolerance proteins that reduce bacterial killing within phagolysosomes.[17 18] Colonic mucosal DAEC isolates from CD and CRC patients are shown here also to possess *htrA* and *dsbA* needed to complete the AIEC phenotype.

UPEC with the DAEC phenotype have previously been shown to invade epithelial cells *in vitro* but it has been uncertain whether or not they invade fully differentiated intestinal cells.[32] It should be noted that the same also applies to AIEC isolates from CD, which have not been convincingly seen within intestinal epithelial cells in human mucosal samples. Indeed, even bona fide intestinal pathogens such as *Salmonella* spp., *Shigella* spp., Mycobacteria and Cholera vibrios require, as their initial portal of entry, translocation across the specialised M cells that overlie Peyer's patches and lymphoid follicles.[33] It seems likely that for CD-associated *E coli* also, initial invasion occurs *in vivo* via M cells, a process that probably requires possession both of LpfA, shown here to be common among CD DAEC, and FimH.[19 34] This accords with evidence that Peyer's patches in the distal ileum and lymphoid follicles in the colon are the sites for the earliest lesions (aphthoid ulcers) in CD.[34 35] It is intriguing that Peyer's patches and lymphoid follicles affected by early Crohn's lesions have surrounding neovascularisation that may be seen as a 'red ring sign' on colonoscopy[36] and enhanced by previous fluorescein injection,[37] a phenomenon that could plausibly reflect angiogenesis driven by Afa-positive *E coli*.

Table 7 Presence of *afaC*+, *pks*+ *E coli* in patients with CD, colitis and colon cancer compared with controls (using total number of *E coli* as the denominator)†

	afaC+, *pks*+	*afaC*−, *pks*−	p Value*
CD	13	58	NS
CRC	35	85	0.0119
UC	17	34	0.0082
Controls	3	36	

*p Values obtained using χ^2 test (Yates-corrected).
†Presence or absence of *afaC* and *pks* is based on PCR assay.
afaC, gene encoding afimbrial adhesin outer membrane usher protein; CD, Crohn's disease; CRC, colorectal cancer; *pks*, polyketide synthase gene complex; UC, ulcerative colitis.

Mucosa-associated AIEC have been reported particularly in the ileum of CD patients,[2 3 14] although they also occur in the colon,[4–6] and studies that include ileal and colonic samples from the same individuals have shown mucosa-associated *E coli*, if present, throughout the terminal ileum and colon.[7] Their adhesion to ileal mucosa requires overexpression of CEACAM6, occurring in response to inflammation.[38 39] The 'paradigm' ileal AIEC LF82,[2] is *afaC* negative,[16] although other ileal isolates tested were found to be *afaC* positive. It seems likely from the studies presented here, that DAEC possessing *afa-1* may be better suited to colonisation of the colonic environment.

Members of the Afa/Dr family commonly use a glycosylphosphatidylinositol-anchored protein, the decay-accelerating factor (DAF), as a cellular receptor.[20] DAF is apically localised in differentiated Caco2 cells.[40] Afa/Dr adhesins also bind variably to CEACAM-1, CEACAM-5 (CEA) and CEACAM-6.[41] DAEC binding induces the recruitment of DAF and CEA family receptors around adhering bacteria by a lipid raft-dependent mechanism, which initiates internalisation and cell signalling.[29 40–43] Both CEACAM-6 and DAF are upregulated in IBD and CRC,[44 45] and thus could favour Afa/Dr-expressing *E coli* colonisation. DAEC infection of the human colonic T84 cell line promotes IL-8 release and neutrophil transepithelial migration, which in turn induces TNFα and IL-1β-dependent upregulation of DAF.[46]

It has been shown previously that DAEC are partly resistant to phagocytosis by neutrophils.[47] Recombinant *E coli* expressing Afa/Dr adhesins, including Afa-1, Afa-III, Dr and F1845, adhere to neutrophils but remain extracellular.[48] The present study suggests that Afa also confers resistance to uptake by macrophages.

It is interesting that the presence of Afa-1, although conferring the ability to invade some epithelial cell lines, does not confer the ability to translocate across M cells. Although this has been shown here using an *in vitro* M-cell model, we have previously shown excellent correlation between results obtained in this model for human colonic mucosa-associated *E coli* and their ability to translocate across human FAE in ileal explants cultured in Ussing chambers.[28] M cells are phagocytic *in vitro*, even for inert particles, and it may be that translocation across M cells bears more relationship to phagocytosis than to invasion. Translocation is dependent not only on the possession of LpfA but also on interaction between FimH and its receptor, GP2, selectively expressed by M cells.[19 34] The majority of the *E coli* isolates shown here to possess *afaC*, *dsbA*, *htrA* and *lpfA*, also possess *fimH*. It is intriguing that circulating anti-pancreatic antibodies found in CD patients have GP2 as their epitope.[49] It seems plausible that a combination of bacterial protein and attached GP2 may be presented as foreign antigen in a manner analogous to the co-presentation of gliadin peptide and tissue transglutaminase in the development of the celiac-associated autoantibody.

Our data show a higher prevalence of colonic mucosal *afaC*-positive *E coli* in patients with ileal and/or colonic CD and CRC than in controls. Previous studies of *E coli* isolates from

patients with CD did not find significant differences in *afaE-3* or *afa/draBC* prevalence in CD patients nor in *afa/draBC* frequency between AIEC/non-AIEC isolates.[10 11] However, the same research group recently reported *afaE-3* association in approximately 24% of *E coli* isolates of newly diagnosed CD patients.[50] One major difference with those studies is the choice of primers used to screen isolates. The primers used in the present study target all *afa* strains, irrespective of the *afaE* subtype.[25]

DAEC not only promote VEGF secretion by epithelial cells but also induce epithelial–mesenchymal transition (EMT)[29] implicated in carcinoma progression.[51] EMT contributes to intestinal fibrosis in a mouse model of CD, and EMT markers have also been detected in Crohn's fistulas.[52 53] VEGF/VEGFR2 signalling similarly links between inflammation and colitis-associated cancer and promotes tumour growth *in vivo*.[30]

The association between colonic mucosal DAEC and CRC adds to growing evidence linking bacteria with CRC pathogenesis. We have previously speculated that bacterial–epithelial interactions might be particularly important in progression from dysplasia to cancer.[54] Dysplastic mucosa is usually goblet cell depleted and lacks overlying mucus. Moreover, the underlying glycocalyx is sparse. It is therefore much easier for bacteria to gain direct contact with the mucosal surface, a location that is relatively sterile in the normal colon. This would allow interaction between bacterial components and Toll-like receptors, with subsequent downstream signalling via MyD88 to nuclear factor κ B activation. Epithelial nuclear factor κ B activation, rather than histological inflammation, has been implicated as the mechanism for inflammation-associated CRC,[55] moreover MyD88-deficient mice cross-bred onto Apc^{min} mice show markedly reduced tumour formation.[56] The ability of *pks*-expressing *E coli* to damage DNA probably makes these bacteria particularly dangerous to the host if they become established in close contact with the colonic epithelium. If epithelial-associated bacteria such as DAEC play a causative role in CRC and CD then dietary consumption of soluble plant fibres that prevent mucosal recruitment of bacteria[5 28] may be protective against both conditions.

The strong association between colonic mucosal *afa*-positive DAEC and both CD and sporadic CRC suggests a possible role for DAEC in the pathogenesis of both conditions, but does not imply that the mechanisms involved will be the same for both. Therefore, co-expression of *lpfA*, important for M-cell translocation, is relevant to CD but unlikely to be relevant to CRC. Co-expression of the genotoxicity island *pks*, possession of which confers the ability to induce breaks in double-stranded DNA, is relevant to CRC but not obviously to IBD. Therefore, the possible mechanisms for *E coli*-induced carcinogenesis may be independent from any effects on IBD pathogenesis. The link with Afa expression probably relates to the propensity for DAEC to colonise the colonic mucosa although studies have yet to be performed to address this directly. Intervention studies will ultimately be needed to assess the role of DAEC in the pathogenesis of CD and CRC.

Contributors BJC and JMR contributed equally. BJC, JMR and CW obtained funding and with JRM, designed research. AA, BJC, CC, CLR, FS, MKF, MP-H, PKF, PK and NH performed experiments. BJC, CW, MP-H, MKF, JRM and JMR performed analyses and interpretation of data. BJC, JMR and MP-H drafted the manuscript, with critical revision by all authors.

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Competing interests JMR is/has been a member of advisory boards for Atlantic, Procter & Gamble and Falk, has received speaking honoraria from Abbott, Falk, Ferring, Glaxo Smith Kline, Procter & Gamble and Schering Plough and, with the University of Liverpool and Provexis PLC, holds a patent for use of a soluble fibre preparation as maintenance therapy for Crohn's disease. BJC has received a speaking honorarium from Amgen Inc.

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Data sharing statement We affirm that all data necessary for a reader of *Gut* to understand and evaluate the conclusions of the paper will be archived in an approved database and made available to any reader. After publication, all reasonable requests for materials and data will be fulfilled. There is a University of Liverpool MTA regarding the human mucosal *E coli*. We can confirm that to date all requests from researchers within the international scientific community for isolates from the Liverpool archive have been granted and materials supplied.

Accession number The nucleotide sequence of the *afa-1* operon from *E coli* HM358 has been submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/); accession number JN688153.

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