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DOMAIN 8 PATHOGENESIS

Characterizing the Pathogenic Potential of Crohn's Disease-Associated Adherent-Invasive *Escherichia coli*

MEGAN T. ZANGARA,[a] LENA DARWISH,[a] AND BRIAN K. COOMBES[a,b,c]

[a]Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada
[b]Michael G. DeGroote Institute for Infectious Disease Research, Hamilton, Ontario, Canada
[c]Farncombe Family Digestive Health Research Institute, Hamilton, Ontario, Canada

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Address correspondence to Brian K. Coombes, coombes@mcmaster.ca.
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ABSTRACT The microbiome of Crohn's disease (CD) patients is composed of a microbial community that is considered dysbiotic and proinflammatory in nature. The overrepresentation of *Enterobacteriaceae* species is a common feature of the CD microbiome, and much attention has been given to understanding the pathogenic role this feature plays in disease activity. Over 2 decades ago, a new *Escherichia coli* subtype called adherent-invasive *E. coli* (AIEC) was isolated and linked to ileal Crohn's disease. Since the isolation of the first AIEC strain, additional AIEC strains have been isolated from both inflammatory bowel disease (IBD) patients and non-IBD individuals using the original *in vitro* phenotypic characterization methods. Identification of a definitive molecular marker of the AIEC pathotype has been elusive; however, significant advancements have been made in understanding the genetic, metabolic, and virulence determinants of AIEC infection biology. Here, we review the current knowledge of AIEC pathogenesis to provide additional, objective measures that could be considered in defining AIEC and their pathogenic potential.

KEYWORDS Crohn's disease, adherent-invasive *E. coli*, host-pathogen interactions, innate immunity, pathogenesis

The human gastrointestinal tract is home to trillions of microorganisms, known as the microbiome, that aid in the digestion of food, help train and develop the host's immune system, and protect against invading pathogens and other noxious substances (1–3). The microbiome has developed a mutualistic relationship with its host, where in return it is able to readily scavenge nutrients from luminal contents while residing in a relatively stable environment (1). However, some microbes in the gut can change their relationship

with their host from commensal to disease modifying, particularly when the gut ecosystem is disrupted by environmental or host-derived factors that disorganize the microbial structure or impair host defenses. In these situations, the dysbiosis that is established is usually comprised of a less diverse microbial community and dominated by a few species that can rapidly outcompete other microbes (4). Once established, this dysbiotic state can become stable and contribute to chronic inflammation and intestinal damage that is difficult to resolve.

Crohn's disease (CD), a subtype of inflammatory bowel disease (IBD), is caused by still relatively obscure perturbations in the complex interactions between the host immune system, microbiota, and environmental factors, which manifest as chronic inflammation of the gastrointestinal tract (5, 6). To date, more than 240 gene loci have been correlated with IBD risk via genome-wide association (GWA), along with external factors such as smoking, stress, infections, and diet (7). Alterations to the composition of the microbiome are common in CD patients. This dysbiotic state is thought to play a primary role in disease activity, as fecal diversion can aid in disease control in a subset of CD patients (8). The microbiome profile of patients at disease onset displays increased abundance of *Enterobacteriaceae*, *Pasteurellaceae*, *Veillonellaceae*, and *Fusobacteriaceae*, as well as decreased abundance of *Erysipelotrichales*, *Bacteriodales*, and *Clostridiales*. Although no bona fide pathogen has been defined as causal in CD pathogenesis (9), in 1998 Darfeuille-Michaud discovered *Escherichia coli* strains in ileal biopsy specimens of CD patients that displayed increased adherence and invasion of intestinal epithelial cells. These CD-associated *E. coli* strains did not encode the common virulence factors found in the well-known pathogenic *E. coli* strains associated with acute infectious gastroenteritis (10) and therefore were called adherent-invasive *E. coli* (AIEC) to reflect their unique nature. AIEC strains have now been associated with CD in numerous countries around the world, suggesting their emergence and overrepresentation in the CD microbiome could have disease relevance (11). Subsequent research determined that CD-associated *E. coli* strains can interact with carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) receptors that are overexpressed on epithelial cells in CD patients, can survive in both murine and human macrophages and epithelial cells, and can elicit inflammatory cytokines from infected macrophages (11–14).

Despite extensive strain sequencing and comparative genomic studies of AIEC, no definitive genetic signature has been able to delineate the AIEC pathotype from non-AIEC or other commensal *E. coli* strains (15). As a result, the current method to define a clinical isolate as AIEC relies on a limited set of *in vitro* phenotypic traits that have proven to be quite heterogeneous among AIEC strains (16, 17). This review aims to provide an update on current knowledge of AIEC pathogenesis with a view to identify areas where AIEC classification could be improved. Our goal is to provide additional, objective measures that could be considered in the current screening process to increase the sensitivity and specificity of AIEC identification.

HISTORICAL CLASSIFICATION OF AIEC

AIEC was first identified 25 years ago in a biopsy specimen collected from an ileal CD patient. In this first report, 65% of samples from chronic ileal lesions and 100% from early lesions contained *E. coli* (10), and this species was the numerically dominant bacterium identified. In samples from healthy individuals included as controls, *E. coli* was found in only 10% of samples, indicating a clear enrichment in the CD patient population (10). The *E. coli* strains isolated in this first study showed increased adhesion to Caco-2 intestinal epithelial cells (IECs) but lacked the virulence genes associated with epithelial cell attachment in pathogenic *E. coli*, namely, the locus of enterocyte effacement (LEE) (18), which encodes a type 3 secretion system (T3SS) (10). Compared with *E. coli* strains categorized as enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroaggregative (EAggEC), enterohemorrhagic (EHEC), and diffusely adhering (DAEC), one of the CD-associated *E. coli* strains, LF82, was as invasive into HEp-2 cells as EIEC and EPEC strains (11). Intracellular AIEC cells were able to survive at least 24 h and could be found in both vacuoles and free in the cytoplasm (11, 19). The invasive phenotype of LF82 was later determined to be dependent on host cell microfilament and microtubule dynamics, as well as type 1 pili on the bacterium (11, 12, 20). Further assessment of LF82 infection biology revealed it was able to replicate within the J774 macrophage cell line and induce the production and release of tumor necrosis factor alpha (TNF-α) from infected cells without causing cell death (13).

AIEC has a high degree of association with CD. A meta-analysis of 12 studies revealed the prevalence of

AIEC among CD patients was ~30%, compared to 9% in non-IBD controls, with an odds ratio (OR) for prevalence of AIEC in CD patients of 3.27 (95% confidence interval [CI], 1.79 to 5.9) (21). A second systemic review and meta-analysis that included 13 case-control studies came to similar conclusions (22). Due to the limited sampling of the intestinal environment by biopsy and the technical challenges associated with sampling the small intestine, these prevalence numbers are almost certainly underestimated. CD patients express increased levels of CEACAM6 on the apical surface of ileal epithelial cells, which serves as a receptor for AIEC adhesion through bacterial FimH in at least some AIEC strains (14). This may explain the increased association of AIEC with CD in some cases; however, one study did show that LF82 did not display increased adherence to CD-derived tissue samples *in vitro* compared to healthy control specimens (23). This suggests that the adhesion landscape of AIEC to host tissues is likely more complex, particularly *in vivo*. Indeed, some AIEC strains, including NRG857c, colonize wild-type (WT) mouse lines with high proficiency despite rodents lacking the CEACAM family of receptors (24).

The method used to classify *E. coli* isolates as AIEC relies on an *in vitro* phenotyping scheme developed nearly 25 years ago. Following exclusion of frank pathogenic *E. coli* types that can be readily identified through signature gene content, AIEC strains are defined based on (i) their ability to adhere (>1 bacterium per cell) and invade (≥0.1% of the original inoculum) IECs, (ii) their survival and/or replication within macrophages, and (iii) their ability to induce TNF-α release by infected macrophages (16, 17). The macrophage cell line typically used is J774, which was used in the original publications that defined the AIEC pathotype. Since the discovery and first description of AIEC strain LF82, additional AIEC strains from both IBD patients and individuals without IBD have been characterized using this method of classification (15, 25–30). Clinical isolates of AIEC have been investigated to various degrees in cell culture models and mouse models to better understand their origin, defining features of the pathotype, infection biology, and role in disease activity.

GENETIC SIGNATURES AND *IN VIVO* ESSENTIAL GENES

A defining genetic signature of AIEC has proven elusive. This presents challenges for the rapid and reliable classification of new AIEC strains, as well as for the identification of AIEC carriers who would benefit from a targeted therapeutic strategy. A recent comprehensive review and commentary (31) nicely articulated the difficulty in defining a genetic signature of the AIEC pathotype, concluding that although progress has been made in identifying candidate virulence factors through comparative genomics, what is lacking is a full understanding of the host-pathogen interactions that govern AIEC infection biology to derive more complete meaning from genomic and transcriptomic studies.

A comparative genomics analysis of three AIEC strains and three non-AIEC strains that had matching sequence types, phylogenetics, and virulence genes identified putative single nucleotide polymorphisms (SNPs) that appeared unique to AIEC (32). Based on this, three polymorphic regions were used to build a binary logistic regression-based model to predict the AIEC phenotype (32). The model was then applied to a collection of 22 AIEC and 28 non-AIEC strains and achieved 82% specificity, 86% sensitivity, and 84% accuracy in phenotype prediction (32). However, a follow up study using this genomic model revealed the model was heavily biased by the geographical location in which a strain was isolated (33). The strain collection used to build and validate the model contained strains isolated in Girona, Spain, whereas the validation study included strains isolated from France, Chile, Mallorca (Spain), and Australia (33). Although in the original Girona-based strain collection the three defining SNPs were differentially distributed between the AIEC and non-AIEC strains, that feature was not present in strains from other countries. In testing the model with more geographically dispersed strains, the specificity of the algorithm decreased to 75%, the sensitivity decreased to 45%, and the overall accuracy dropped to 61% (33). Interestingly, if AIEC isolates were from geographically close regions (i.e., Girona and Mallorca, Spain), the prediction model retained a high level of accuracy (81%) and specificity (82%) (33), suggesting that there are regional differences that influence AIEC strain-level diversity in the population. Whether this is driven by host genetics, regional host-to-host transmission, or other local environmental factors that impose selection is unknown.

A better understanding of AIEC infection biology will likely aid in identification of AIEC genes under host selection and could help reveal a more discriminating genetic signature. To advance this idea, the AIEC genetic determinants contributing to colonization of the murine gut were

screened in the NRG857c AIEC strain, using a highly saturated transposon mutant library of over 600,000 mutants (34). Genes essential for in-host survival were determined by colonizing C57BL/6N mice with the mutant pool and sequencing the surviving mutants recovered in the cecal contents 3 days postinfection (34). The abundance of each mutant was determined relative to the input inoculum using deep sequencing and the ConARTIST analytic pipeline (34). Among the mutants depleted *in vivo*, approximately 50% harbored mutations in metabolic genes (see Metabolic Adaptations of AIEC in the Intestinal Environment), as well as multiple mutants in a type 4 secretion system (T4SS) (see Characterization of a Unique Virulence Profile) (34). Interestingly, *in vivo* RNA-seq experiments revealed the same genes to be transcriptionally upregulated during infection by wild-type AIEC, indicating that their expression *in vivo* confers a fitness advantage that promotes AIEC host colonization.

A longitudinal metagenomics case study of one CD patient who consistently presented with levels of *E. coli* at 100-fold greater than typically found in healthy controls revealed that *E. coli* strain dominance is highly dynamic (35). Over a 3-year period, 27 stool samples and corresponding metadata were collected and analyzed; at any given time point, one *E. coli* strain dominated in abundance, but dominance switched between 7 different strains in waxing and waning periods (35). Using *de novo* metagenomic assembly and functional annotation using Prokka, draft assemblies of the dominant strains were analyzed for phylogeny, sequence type, distribution of virulence factors, and metabolic networks. One strain, ST1, was isolated from a sample collected during peak inflammation, and was predicted to share many features of AIEC strains LF82 and NC101, while the other strains displayed a diverse range of gene contents (35). This led the authors to hypothesize that the shifting dominant strain correlates with fluctuations in disease activity, differentially contributing to the progression of disease. More longitudinal studies within individuals are necessary to help sort out the biological significance of *E. coli* strain displacement on disease activity.

The most readily utilized method of characterizing the microbial profile from patient samples is sequencing the variable region of the 16S rRNA gene, but this has its limitations (36). While it is an effective method to profile samples at the family and genus level, differentiating between closely related species or strains is not possible (36). Shotgun metagenomic sequencing, as used in the case study mentioned above, allows for a higher level of taxonomic resolution and additional functional profiling but is more costly and involves a more complex bioinformatics pipeline. Recently it has become possible to sequence the whole rRNA operon, inclusive of all 9 variable regions of 16S, the internal transcribed spacers (ITS), and 23S (36, 37). Using a collection of 4 AIEC strains and 4 non-AIEC strains of *E. coli*, all from the B2 phylogroup and isolated from IBD, non-IBD, and murine sources, it was demonstrated that sequencing longer regions of 16S, ITS, and 23S increased the sensitivity and specificity of strain detection, with an average accuracy of 87.3%, compared to accuracies of 35% and 27% when only the V1-V2 or V3-V4 hypervariable regions, respectively, were sequenced (37). Even in a ratio of 0.5% bacterial to 99.5% human DNA, full-length rRNA sequencing was able to identify specific bacterial strains (37). This method is useful for identifying the presence of known strains, and the accuracy of detection even with a low concentration of target DNA suggests that this may be a viable resource for determining if a patient is colonized with AIEC, allowing for better-informed decisions regarding clinical care.

CHARACTERIZATION OF A UNIQUE VIRULENCE PROFILE

Interaction with intestinal epithelial cells. One of the first comprehensive studies to investigate AIEC adherence and invasion of IECs identified type 1 pili (T1P) as a critical virulence factor in AIEC strain LF82 (12). This was done using a Tn-*phoA* mutant library that can enrich for membrane-associated or secreted protein targets under selection owing to the alkaline phosphatase activity of the fusion. Screening this library in a gentamicin protection assay in Intestine-407 cells identified several mutants in the *fim* operon as being defective for cellular invasion (12). The *fim* operon encodes the subunits for T1P that enable *Enterobacteriaceae* to colonize host epithelial surfaces, and mutations in any of components involved in pilus biogenesis impair host colonization in several Gram-negative pathogens (12, 38, 39). Functional analyses and transmission electron microscopy revealed that T1P were essential for AIEC invasiveness of IECs in a manner that requires host cell actin microfilaments and microtubules to

induce membrane protrusions that surround the bacterium at the site of adhesion and facilitate its uptake into an endocytic vacuole (ECV) within the cell (11, 12). As previously mentioned, the AIEC FimH adhesin contains unique amino acid substitutions that enhance its binding to the host glycoprotein CEACAM6 expressed on the surface of IECs (14, 40, 41). As a feature of its intracellular infection strategy, a proportion of AIEC cells subsequently lyse and escape the ECV and survive intracellularly by replicating in the cytoplasm in a process that is currently poorly understood (11). Since the initial phenotyping of AIEC, the determinants of adhesion, invasion, and survival within IECs remain an active subject of investigation. Thus far, no singular virulence factor appears specific to AIEC; rather, these bacteria employ canonical strategies common in other Gram-negative bacteria to facilitate host colonization, including pilus-mediated adhesion (41), flagellar-based motility (42), and evasion of host defense pathways (43). Components of the host intestine, including bile acids and mucins, seem to promote increased expression of flagellar genes in AIEC, allowing for hypermotility and thus increased invasion of the gut mucosa (42, 44). Flagellar gene expression in AIEC appears to be coregulated with T1P and potentially other virulence factors. In *E. coli* LF82, mutation of the flagellar regulator *fliA* interrupted the cyclic di-GMP signaling cascade and abrogated expression of T1P through an unknown mechanism (20, 45). Flagellar regulation may also be implicated in regulating accessory factors that help promote the invasion process since complementation of T1P in *trans* in Δ*fliA* mutants did not fully restore invasiveness to that of WT *E. coli* LF82 (45). Regulatory roles for flagellar components in controlling accessory virulence factors have been described in other pathogens, including uropathogenic *E. coli* (UPEC) (46–49), suggesting that gene regulation pathways may be under host selection, as has been described in other human pathogens (50). Supporting this idea, adhesion to host epithelial cells induces expression of the T4SS involved in biofilm formation (34), suggesting that unknown host cues reprogram AIEC transcription toward an adhesive phenotype that has selective benefits for the bacterium. Greater understanding of the transcriptional profile of AIEC across the biogeographical landscape of the gut should be a priority for future research to gain a comprehensive understanding of AIEC infection biology throughout the gut.

Other AIEC proteins in addition to T1P mediate invasion of IECs, including the surface lipoprotein NlpI (51), the periplasmic oxidoreductase DsbA (52), and YfgL, which is a lipoprotein implicated in *E. coli* LF82 invasiveness via outer membrane proteins (OMPs) and outer membrane vesicle (OMV) release (53). Clinically, the link between CD severity and elevated levels of antibodies against *E. coli* OmpC makes OMPs and OMV production in AIEC interesting candidates to pursue as mechanisms of virulence (54–56). Initial work identified the EnvZ/OmpR two-component system as being involved in OmpC-dependent activation of the RpoE regulatory pathway that modulates expression of T1P, flagella, and other unknown virulence factors (57). AIEC may also take advantage of aberrant expression of the host endoplasmic reticulum stress protein Gp96 on the surface of epithelial cells in CD patients as a binding site for OMVs to promote AIEC invasion (58). The mechanism raises the possibility that OMVs might serve to deliver additional bacterial payloads into target cells to modify host cell processes, as shown in other pathogenic *E. coli* strains (53, 59–62). Quantitative mass spectrometry has been used to study the protein content of AIEC and ETEC OMVs compared to reference commensal strain *E. coli* MG1655 in search of differential protein expression that may be related to virulence (63). Under culture conditions that approximate the small intestinal environment, AIEC OMVs and culture supernatant fractions showed unique enrichment of proteins related to iron acquisition (FhuA and EfeB), oxidative stress (SodA and RclR), metabolic cascades (AdhE, FabF, TyrS, and AspC), the T4SS (TraV), and fimbriae (FimA) (63). In addition, OMP sequences and expression patterns have been compared between AIEC strains and to non-AIEC strains during *in vitro* infection of IECs (64). It was found that there were no OMP sequence variants that could be specifically assigned to AIEC, although certain amino acid substitutions in OmpA, OmpC, and OmpF correlated with increased AIEC adhesion and invasion indices (64). In agreement with previous findings, AIEC upregulated OmpA expression upon association with IECs relative to the supernatant fraction (64). In comparison, non-AIEC strains were found to upregulate all three of OmpA, OmpC, and OmpF when associated with IECs (64). However, the analysis of OMP expression in the IEC-associated fraction did not discriminate between adhered and intracellular bacteria, leaving to question whether differences in the degree of invasion contribute to regulation of OMP expression (64). With respect to other secreted virulence factors, *E. coli* LF82 releases the serine protease Vat-

AIEC, a mucolytic enzyme that allows AIEC to gain proximity to IECs by degrading the protective mucus layer (65). Interestingly, the highest levels of Vat-AIEC secretion occurred *in vitro* at 7.5 pH in the presence of bile salts and mucins, all conditions that would be present in the ileal environment (65). This emphasizes the need for host-relevant models and prompts further investigation into secreted virulence factors.

Long polar fimbriae (LPF) are used by many enteroinvasive bacteria to invade the follicle-associated epithelium (FAE) lining Peyer's patches (PPs), where microfold (M) cells can be accessed as entry points into the lamina propria (66–68). Several AIEC strains contain an *lpf* operon (66, 69) that can confer tropism to PPs by binding to glycoprotein 2 (GP2) on the surface of M cells (66). GP2 is also recognized by FimH of T1P (70), creating a seemingly redundant system which implies that M cell transcytosis could be a key entry point for AIEC infiltration of the lamina propria. It is important to note that not all AIEC strains harbor the *lpf* operon (71). Similarly, other putative virulence genes are differentially distributed among AIEC strains (71). Given that even highly similar AIEC isolates colonize preclinical mouse models to various degrees (24), it is reasonable to postulate that subtle differences in gene content and regulatory pathways translate to clinically meaningful differences in how AIEC may alter disease activity. Indeed, within-host evolution experiments have revealed the emergence of stable, phenotypically distinct AIEC subpopulations that occupy distinct niches within the gut (42). As more becomes known about the infection strategies used by AIEC across the varied biogeography of the gut, it may become appropriate to define AIEC subclasses based on their cellular tropism, their location within the length of the gastrointestinal tract, or their lifestyles within the gut environment.

Persistence and survival in intestinal macrophages.

A defining property for nearly all intracellular pathogens is evasion of immune recognition. Upon its discovery, one of the first lifestyles associated with AIEC was occupation of the intracellular niche within macrophages (13). Thus far, the genetic determinants mediating intracellular survival and replication of AIEC within macrophages and the relative contribution of this bacterial population to disease course remain largely unknown. An AIEC LF82 transposon mutant library guided preliminary progress in cell culture models to identify genes that are essential for intramacrophage survival and replication (52, 72). This early work found the high temperature requirement A (HtrA) stress protein and disulfide bond formation A (DsbA) oxidoreductase protein to be important during macrophage infection by reducing oxidative and envelope stress in AIEC cells growing in the phagolysosome microenvironment (52, 72). More recently, the IbeA invasin of the *ibeRAT* operon, found in pathogens of the *E. coli* B2 phylogenetic group, was identified as playing a major role in intramacrophage survival of AIEC strain NRG857c, as well as epithelial and M cell invasion *in vitro* (73). However, the absence of *ibeA* in AIEC strain NRG857c does not seem to impair invasion of intestinal tissue *in vivo* (73), indicating that other colonization factors are involved within the gut milieu.

The survival strategies that underpin AIEC persistence constitute the other side of AIEC virulence (24), as persisters across several bacterial species represent a problematic population with respect to host health and treatment (74). After engulfment by macrophages, bacterial stress pathways, including the stringent response and SOS pathway, can act as secondary signals to slow bacterial growth (75). This process creates a heterogeneous population of either replicating AIEC or a nonreplicating persister population that exhibits increased stress tolerance to components of the innate immune system and antibiotics (75). Following the stress-induced halt in bacterial growth, resumption of AIEC multiplication coincides with the production of an extracellular matrix and a transcriptomic profile consistent with the transition from a planktonic to a biofilm-like state (76). The result is the formation of intracellular biofilms that support long-term persistence inside macrophages, likely by way of iron capture or evasion of host innate defense systems (76). Intracellular bacterial communities have been described for other bacterial pathogens as well, most notably uropathogenic *E. coli* (UPEC), but also including *Pseudomonas*, *Borrelia*, and *Moraxella* (77).

AIEC can readily form biofilms, organizing into a densely packed bacterial population within polysaccharide polymers that are more resilient to antibiotics and immune-based clearance (78, 79). Extracellular biofilms of AIEC have been demonstrated in a preclinical mouse model (34) and may be promoted by dietary food additives (80) and microbiome-derived propionic acid (81). In fact, 90 to 95% of IBD patients exhibit high densities of mucosa-located bacteria compared to 35% of healthy individuals,

and biofilm density was 100-fold greater in IBD patients than in controls (82). Consistent with this, a higher proportion of AIEC strains are strong biofilm producers *in vitro* compared to commensal *E. coli* (78). The RpoE regulatory pathway, which responds to the high osmolarity of the gut, has been implicated in indirect regulation of T1P and flagella among other unknown virulence factors and also directly promotes biofilm formation in *E. coli* LF82 as opposed to commensal *E. coli* (57, 83). *In silico* screening for downstream genes of the RpoE regulon that facilitate AIEC virulence identified 12 genes specific to *E. coli* LF82, pinpointing the *waaWVL* operon as a central group of genes for biofilm formation in AIEC both *in vitro* and *in vivo* (84). Interestingly, disruption of the *waaWVL* operon shows that these enzymes are dispensable for growth and biofilm formation in APEC, UPEC, and *E. coli* strain HS, whereas its disruption in AIEC is lethal, and when a modified mutant was created to circumvent lethality, AIEC biofilm formation was also severely abrogated (84).

The recent use of transposon insertion sequencing (TIS) has facilitated a more comprehensive understanding of so-called "*in vivo* essential genes" in AIEC (34). Elhenawy and colleagues found that AIEC employs a T4SS for biofilm formation in the gut that increases fitness within the host (34). T4SSs are nanomachines that span the bacterial cell membrane and express surface pili which can be used for DNA transport, protein secretion, and biofilm formation. In a mouse model of AIEC colonization, a T4SS mutant was not able to colonize or persist in the gut like WT AIEC (34). Immunofluorescence microscopy corroborated these observations by showing that T4SS mutants were unable to aggregate and form microcolonies on the surface of IECs, strongly suggesting that T4SS-mediated biofilm formation promotes stable AIEC colonization of the host (34). Therefore, formation of biofilms may be a critical component of AIEC persistence. Interestingly, the *tra* operon encoding the T4SS was significantly enriched in the microbiota of CD patients compared to that of healthy controls (34). However, *traD* was only present in 12 out of 40 analyzed AIEC strains (34), suggesting the presence of additional regulators of biofilm formation to allow AIEC without a T4SS to be competitive. Although the biological significance of extracellular biofilms and intracellular aggregates of AIEC on disease course is not well defined, these growth phases doubtless contribute to a persistent AIEC populations that can endure in the inflamed gut environment and expand when conditions become favorable to do so. To better understand the role of AIEC in Crohn's disease activity, research on AIEC persistence and tolerance must continue to be prioritized.

ANTIBIOTIC AND ANTIMICROBIAL PEPTIDE RESISTANCE PROFILES

Antibiotics. Alteration to the intestinal microbiome is a hallmark feature in Crohn's disease and likely has a principal role in disease pathogenesis. *E. coli* strains isolated from CD patients tend to be more antibiotic resistant than *E. coli* strains from non-IBD individuals, which likely relates to antibiotic use among IBD patients as part of their clinical management (85). Resistance to β-lactam antibiotics is more common in AIEC versus non-AIEC strains, but resistance frequency is similar for tetracyclines, aminoglycosides, and quinolones (85, 86). In one study, AIEC strains were resistant to four or more antibiotics on average compared to two antibiotics in non-AIEC strains (85). Multidrug resistance, defined by the proposed international standard as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories (87), was twice as frequent in AIEC strains (85). Thus, many AIEC strains satisfy the definition of "superbugs."

The high prevalence of β-lactam resistance has been linked to either plasmid-mediated AmpC β-lactamases or mutations in the promoter region of chromosomal *ampC* that lead to overexpression and thus high-copy suppression. Analysis of the genome of LF82 revealed 5 different mutations throughout the promoter and chromosomal *ampC* gene. While other strains with β-lactam resistance also showed some of the mutations found in LF82, no mutation was present in all strains (85). Strains with resistance to first-generation cephalosporins also harbored TEM β-lactamases (85, 86). In two studies, one using a collection of 31 AIEC and 56 non-AIEC strains, the genes *sul1* and *sul2* were identified in sulfonamide-resistant strains, and *sul1* was more frequent among AIEC strains (85, 86).

The relationship between patient disease status (CD versus non-IBD) and prevalence of antibiotic resistance in isolated *E. coli* strains is not clear. In one study, resistance to amoxicillin, cefoxitin, chloramphenicol, ciprofloxacin, and gentamicin was observed only in CD-associated strains, while resistance to tetracycline, clarithromycin, and ampicillin was independent (86). Another study showed that resistance to chloramphenicol was found only in CD isolates, in addition to resistance to streptomycin and ampicillin, although this

did not reach statistical significance (88). In a third study, no difference in frequency of resistance to any antibiotic class was observed when strains were stratified by patient disease status (85). One exception to this discrepancy between studies was with rifamycins; Dogan et al. showed that rifaximin resistance was present only in CD-associated strains, Cho et al. showed elevated (but nonsignificant) resistance to rifaximin in both CD and ulcerative colitis (UC) isolates, and Martinez-Medina et al. determined that a higher proportion (71% versus 39%) of CD-associated strains displayed higher MICs against rifampin (85, 86, 88). While this piece of data suggests that there is a lack of correlation between disease status and AIEC antimicrobial resistance, it should be taken into consideration that CD patients are likely more frequently exposed to antibiotic agents. Antibiotics, such as rifaximin, are routinely prescribed to CD patients after surgery to mitigate postoperative recurrence of disease (89). If patients are already colonized to a higher degree with AIEC, antibiotic treatment may serve to select for resistant strains that become the dominant population (21, 22).

In addition to drug-inactivating resistance genes, many bacteria express efflux pumps (EPs), allowing for the export of a large variety of products, including antibiotics, out of the bacterial cell. A survey of EP-encoding genes, using *E. coli* K-12 as a reference, revealed that the AIEC LF82 genome has maintained 19 of the 20 common EPs with high sequence identity (generally >95%), with only *mdtM* being completely absent (90). As AIEC has a multiphasic lifestyle, ranging from extracellular planktonic cells, to adherent biofilm aggregates, to intracellular bacteria in various cell types (91, 92), the expression of EPs was assessed *in vitro* during early epithelial cell and macrophage invasion. During the first 4 h of infection, *emrK*, *mdtJ*, *acre*, *mdtK*, *cusB*, and *mdfA* were highly upregulated, independent of host cell type (90). Expression of other EP genes was cell type dependent, with *fsr*, *mdtL*, and *acrA* upregulated only in macrophages, but downregulated or unchanged in the presence of epithelial cells (90). Conversely, *acrA* expression gradually increased over the early infection period in epithelial cells but was moderately downregulated in macrophages (90). The authors proposed that the differential gene expression of EP genes based on host cell type might be a strategy to subvert specific host cell defenses. Interestingly, deletion of the MdtEF EP reduced AIEC survival in macrophages ~2.5-fold (90), suggesting that in some infection contexts, the EP repertoire of AIEC strains appears to influence intracellular survival, likely through evasion of critical host defense functions of macrophages.

Host-derived antimicrobial peptides. The mucosal barrier of the intestine provides chemical and physical control of gut homeostasis by limiting bacterial encroachment toward the epithelium (93–95). The epithelium exerts physical control over barrier function through cell-cell tight junctions and goblet cell secretion of mucins that assemble into a glycosylated polymer structure in both the small and large intestines (93, 96). Antimicrobial components such as antimicrobial peptides (AMPs) and secretory IgA make up a first line of defense in the gut (94, 95). AMPs are produced and secreted by Paneth cells, a specialized secretory epithelial cell lineage found in the small intestine, and function to disrupt essential functions of bacteria, resulting in replicative stasis or cell death (94, 95). AMPs are concentrated within the mucus barrier at the base of small intestinal crypt, creating an antimicrobial gradient that protects the stem cell niche as well as differentiating epithelial cells in the crypt walls from bacterial encroachment (94, 95). Many pathogens, including some AIEC strains, have acquired resistance to one or more AMPs. Despite high sequence conservation and gene synteny, strain NRG857c, but not strain LF82, displays a high level of resistance to the α-helical cationic AMPs LL-37, CP10A, and CP28, the α-defensin HD5, β-defensin HBD2, and murine MIG (43). The genetic basis for AMP resistance in NRG857c is localized to a small genomic island on an extrachromosomal plasmid that contains antimicrobial peptide resistance locus A, B, and C (*arlABC*). *arlA* is an ortholog to a *Salmonella* and *Pseudomonas aeruginosa* AMP resistance gene, *alrB* is a predicted NAD-dependent epimerase gene, and *arlC* is a paralog of an *ompT* family outer membrane protease gene with specificity for cationic peptide sequences (43). Deletion of the entire genomic island or *arlA* alone results in sensitization of AIEC to Paneth cell secretions, which contain a complex mixture of AMPs (43). Interestingly, a screen of 97 clinical isolates from IBD patients and control isolates from non-IBD individuals revealed that *arlA* and *arlC* were strictly limited to IBD isolates, with 5 strains positive for *arlA* and 4 strains positive for *arlC* (43). One strain analyzed, UM146, was negative for both *arlA* and *arlC*, but showed resistance for LL-37 and CP10A *in vitro*, suggesting other genes may also contribute to AMP resistance in AIEC strains (43).

Another study screened 88 clinical *E. coli* isolates from CD and UC patients and non-IBD controls (40, 24, and 24 isolates, respectively) and determined that only UC isolates, but not CD-derived strains, displayed increased resistance to LL-37 over the control group (88). Notably, all UC strains displayed some level of resistance to LL-37, with none having complete sensitivity (88). This resistance phenotype among the UC isolates may be due to increased omptin protease activity, as 80% of UC strains were positive for omptin activity compared to 63% and 65% of strains in the control and CD groups, respectively (88). A PCR-based targeted screen revealed a higher proportion of UC strains carry the *ompT* gene (79% versus 67% for the control and 58% for CD), and two of these UC strains also harbor an EHEC-like promoter for this gene, which confers elevated protease activity (88). Some AMP resistance mechanisms, like protease inactivation of extracellular AMPs, can have collective good for an otherwise sensitive bacterial population, which may explain why some AIEC strains seem to have acquired AMP resistance mechanisms while others have not.

METABOLIC ADAPTATIONS OF AIEC IN THE INTESTINAL ENVIRONMENT

The designation of AIEC as a CD-associated pathobiont suggests that it has a selective advantage in the inflamed intestinal environment. Indeed, several enteric pathogens that cause acute infectious gastroenteritis have acquired selective advantages through their ability to carry out metabolic processes not commonly found in commensal bacteria. *Salmonella enterica* serovar Typhimurium is a good example of metabolic adaptation, as one of its strategies to competitively thrive in the intestinal mucosa is the expression of a tetrathionate reductase system that allows it to use inflammation-derived tetrathionate in the gut for respiration (97).

AIEC appears to be highly metabolically flexible in the gut environment (Fig. 1). To study this experimentally, Elhenawy and colleagues developed a host-to-host *in vivo* transmission model of AIEC and studied within-host evolution of AIEC over chronic time scales of months (42). After colonizing index mice with the parental AIEC strain NRG857c and allowing AIEC to establish in the gastrointestinal tract, AIEC was naturally transmitted to secondary naive mice via cohousing. This transmission process continued for ~9 months, after which the emergent AIEC lineages were sequenced and phenotyped (42). Overall, the *in vivo* environment selected for several metabolic adaptations, the most prominent being carbohydrate metabolism and inorganic ion transport (42). There was also selection for AIEC with insertion sequences (ISs) that inactivated repressors of the tryptophan operon and the galactitol utilization operon (42). A main finding was the selection for improved use of acetate as a carbon source in host-evolved AIEC that allowed the host-adapted strain to outcompete the parental strain *in vivo* (42). Acetate and other diet- and microbiota-derived short-chain fatty acids (SCFAs) are integral components of the intestinal metabolome for maintenance of gut homeostasis (98). Considering this, AIEC likely evolves to leverage the host metabolome to gain a competitive advantage in colonization. It is particularly interesting that SCFA utilization does not appear to be a selective trait in host-adapted *E. coli* isolated from non-IBD individuals, whereas it is in *E. coli* from IBD patients (42).

In addition to proficiently using acetate as a carbon source, AIEC can also use the SCFA propionate for growth, stress tolerance, and increased virulence (81, 99, 100). Stress tolerance is thought to be, in part, mediated by the incorporation of propionate into the cell membrane (81). In one instance, Ormsby et al. showed that *E. coli* LF82 retrieved from propionate-fed mice adhered to Caco-2 cells and formed biofilms significantly more than *E. coli* LF82 retrieved from mice not exposed to propionate (81). Although *E. coli* LF82 invasiveness did not increase when inoculated into propionate-fed mice, bacteria did become significantly more invasive to Caco-2 cells when exposed to propionate *in vitro*, once again illustrating the necessity for *in vivo* models when characterizing AIEC (81). Another study showed that in the presence of propionate, AIEC isolate ZvL2 exhibited increased survival and replication in macrophages, coinciding with the increased presence of several proteins implicated in pathogenicity. The most prominent among these were OmpA, previously shown to enhance AIEC interaction with IECs, OmpW, an iron-regulated protein necessary for *E. coli* resistance to phagocytic killing, and master virulence regulator PhoP (99). In CD patients, the propionate concentration in the stool was found to be inversely related to ileal *E. coli* abundance in AIEC-positive patients, suggesting either less SCFA-producing bacteria or increased SCFA catabolism, possibly by AIEC (101). In line with this, the same study demonstrated that colonization of mice with *E. coli* LF82 lowered the *in vivo* propionate concentration in the gut by 40%, which seemed to impact levels of inflammation

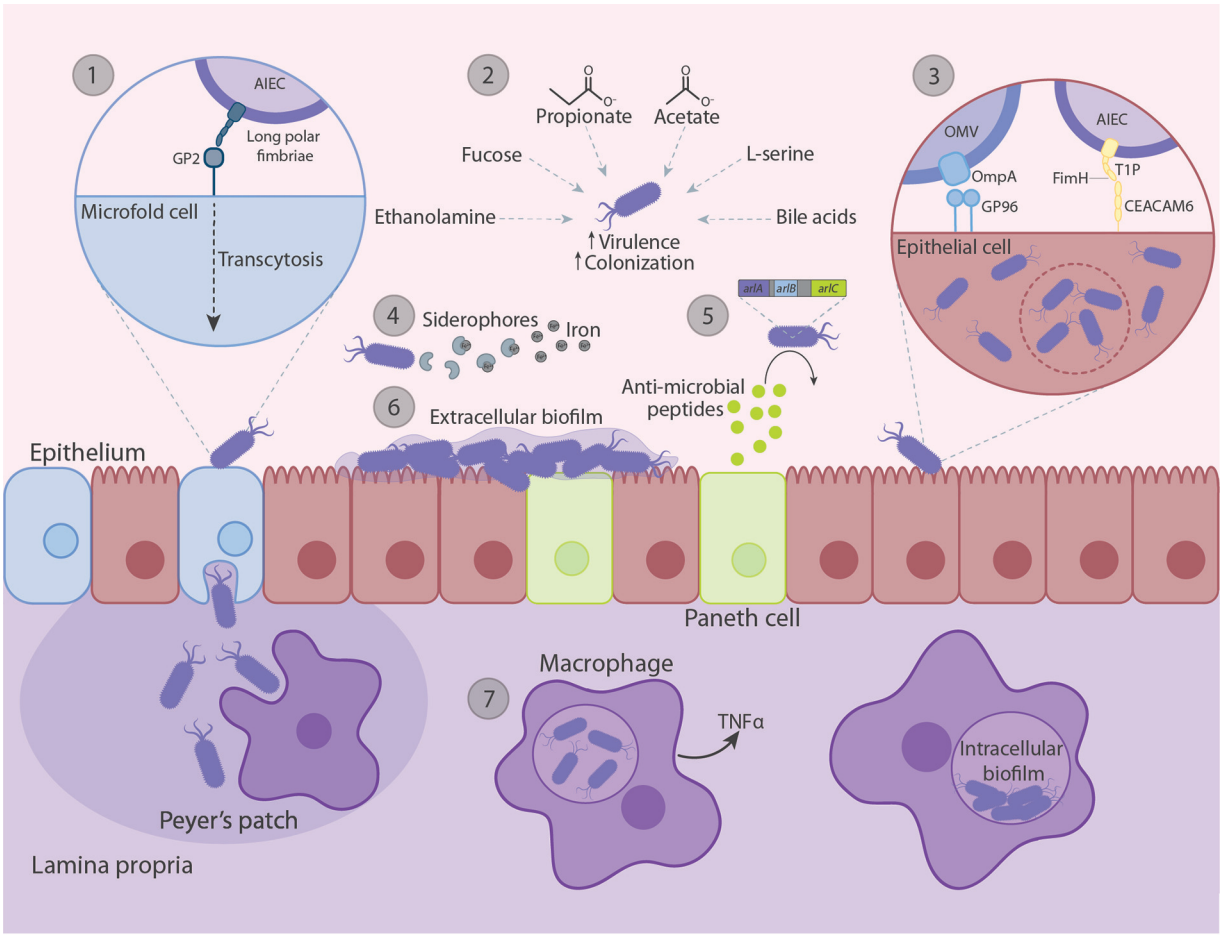

FIG 1 Schematic of AIEC mechanisms of virulence, colonization, and metabolism in the gut. The following seven aspects of these mechanisms are shown. (1) AIEC long polar fimbriae bind cell-surface glycoprotein 2 (GP2) to leverage microfold (M) cells as portals of entry into the lamina propria via transcytosis. (2) Components of the gut metabolome such as short-chain fatty acids, ethanolamine, L-serine, fucose, and bile acids can be detected and metabolized by AIEC, conferring a competitive advantage that allows it to upregulate virulence mechanisms and better colonize the gut. (3) AIEC cells invade IECs using T1P, where the high affinity of the FimH subunit for cell surface CEACAM6 increases invasiveness. Once inside IECs, AIEC cells lyse the endocytic vacuole and survive intracellularly by replicating in the cytoplasm. OmpA as a part of OMVs has a high affinity for cell surface endoplasmic reticulum stress protein GP96, allowing OMVs to potentially deliver bacterial payloads into IECs as a virulence mechanism. (4) AIEC cells are highly proficient at acquiring iron, an essential metabolite, from the intestinal environment through the release of several types of siderophores that evade host nutritional immunity and compete against other bacteria. (5) Some AIEC strains are resistant to host antimicrobial peptides released by Paneth cells due to the plasmid-associated "antimicrobial peptide resistance locus A, B, and C" operon (*arlABC*), allowing better colonization of the gut. (6) AIEC cells are able to form resilient biofilms on the intestinal epithelium that can mediate long-term colonization and persistence within the gut. (7) AIEC cells are engulfed by macrophages once they enter the lamina propria, eliciting an inflammatory response mediated by release of TNF-α. Upon phagocytosis, AIEC cells survive and replicate within the macrophage by persisting in the phagolysosome in both planktonic and aggregate lifestyles.

as well (101). AIEC can also produce propionate itself by way of fucose catabolism through the propanediol utilization (*pdu*) operon. Comparative genomic analysis across a variety of AIEC strains and commensal *E. coli* showed that a complete *pdu* operon was found in 5 out of 8 AIEC strains, whereas it was absent in all 12 of the commensal *E. coli* strains investigated in this study (69). Metagenomic data from 22 CD patients compared to those from 24 healthy participants also showed a significantly higher abundance of *pduC* in CD microbiota, most of which mapped to the *Escherichia* genus (100). The *pdu* operon is otherwise expressed mainly among

pathogens and participates in the metabolism of 1,2-propanediol, a product of fucose catabolism (102–104). While fucose is an essential component of protective intestinal mucus, the resulting by-products of its catabolism, such as propionate, can be used by enteric pathogens for increased colonization (102–104). Initial *in vitro* assays demonstrated that propanediol dehydratase *pduC* expression was indeed stimulated by fucose supplementation and that *pduC* facilitates increased invasion of Caco-2 cells and increased AIEC survival in macrophages (69). In a preclinical mouse model of colitis, fucose catabolism by AIEC strain *E. coli* 2A produced excessive propionate, synergizing with AIEC-derived lipopolysaccharide (LPS) to activate intestinal macrophages and Th17 cells and ultimately inducing T-cell-dependent intestinal inflammation (100).

Iron acquisition is essential for AIEC to overcome colonization resistance and persist in the gut, as it is required for essential enzymatic and metabolic processes (105). AIEC is enriched for a variety of siderophores and iron uptake systems, including enterobactin (*ent*), salmochelin (*iro*), yersiniabactin (*ybt*), aerobactin (*iut*), *Shigella* iron transport (*sit*), and heme iron utilization (*chu*) genes (15, 34, 69). Many of the iron acquisition systems in AIEC encode the production of "stealth" siderophores that can evade host immunity. Under infectious conditions, nutritional immunity in the host releases lipocalin-2 (LCN2), a protein that sequesters catecholate siderophores such as enterobactin and prevents their iron-scavenging function for bacteria, thus limiting bacterial growth (105). Stealth siderophores such as yersiniabactin and salmochelin are able to escape nutritional immunity by being structurally incompatible with the LCN2 binding pocket, conferring AIEC with a competitive advantage against other commensals in the gut under inflammatory conditions (105, 106). Indeed, AIEC strains deficient in either *chu* or *iutA* exhibit decreased *in vitro* survival in macrophages (15, 69). Furthermore, deletion of *iutA* resulted in significantly decreased *in vivo* colonization of AIEC (15).

Amino acids are another class of host-derived molecules that can be uniquely co-opted by microbes. Under disease conditions, *E. coli* LF82 metabolizes L-serine derived from both the host diet and cells (107, 108). Germfree mice monocolonized with *E. coli* LF82 and under chemically induced intestinal inflammation showed that L-serine supports luminal AIEC colonization during inflammation (107). Conversely, the ability to use L-serine was not necessary for AIEC growth under noninflammatory conditions, as shown by the dispensability of the *tdc* and *sda* operons responsible for L-serine utilization in the absence of induced or spontaneous colitis (107). These data suggest that L-serine use is a disease-specific metabolic adaptation of AIEC to inflammation; however, the clinical significance of this in CD patients requires further exploration.

Bile acids are host-derived digestive surfactants that can have secondary activities on pathogens as environmental cues to initiate virulence gene expression (109, 110). AIEC strains appear to have similar bile acid sensing mechanisms that confer a competitive advantage in the gut (111). Transcriptional profiling of AIEC gene expression in the presence of bile salts revealed that the ethanolamine utilization operon (*eut*), along with genes of the citrate degradation and methyl-citrate pathways, were significantly upregulated (111). Ethanolamine in the gut is derived from cell membranes and host diet and is used by many pathogens as a carbon and nitrogen source, as well as some commensals to various extents (112, 113). Bile salts stimulate *E. coli* LF82 to use ethanolamine as a nitrogen source more efficiently *in vitro* and *in vivo* (111). Ormsby et al. used quantitative reverse transcription-PCR (qRT-PCR) to show that expression of *eutS* is increased in AIEC from pediatric CD patients with active disease (114). Bile salts also seemed to stimulate AIEC to accumulate acetyl coenzyme A (acetyl-CoA), a critical intermediate in energy production, through the *eut* operon and genes involved in acetate generation, namely, citrate lyase genes and acetyl-CoA synthetase (111). Increased efficiency in acetyl-CoA formation likely provides an energetic advantage for AIEC to overcome colonization resistance and thrive in its own niche. In a potentially related energetic function, bile salts also induce expression of the *pdu* operon, which produces propionate during its metabolic processes. Propionate can then be used to make propionyl-CoA that can feed the methyl citrate cycle to produce pyruvate, an energetic intermediate that confers competitiveness to *E. coli* LF82 (111). Ormsby et al. showed that these metabolic profiles work in concert, wherein propionate exposure promotes *eut* expression in AIEC, increasing intramacrophage survival as ethanolamine becomes available to the propionate-exposed intracellular bacteria (114). Furthermore, it is important to mention that the study also found that the ethanolamine and propionate metabolic advantage phenotype is not common to all clinical isolates of AIEC. The demonstrated growth advantage in response to pretreatment

with propionate and increasing concentrations of ethanolamine was found in only 50% of the clinical isolates tested (114). Finally, a potentially interesting finding is that among the differentially expressed genes in response to bile salts, 517 encoded proteins of unknown function (111). Knowing that bile salts are a host-derived virulence stimulus, this group of genes may harbor key determinants of AIEC pathogenicity, making bile salts a relevant condition under which AIEC can be studied.

The intestinal metabolome is a product of myriad environmental and microbial factors that are highly variable between individuals and are disrupted in CD patients. Linking metabolite dynamics with microbial composition in the gut will provide new insight into how certain metabolic processes reinforce or repress colonization by microbes. With respect to AIEC, this will uncover host-derived elements that make the gut susceptible to AIEC virulence and potentially new bacterial regulatory pathways governed by external metabolic cues.

CONCLUDING REMARKS

AIEC represents a metabolically versatile bacterial pathotype that is uniquely equipped to thrive in an inflamed host environment. Through a constellation of virulence strategies, AIEC strains can adopt various lifestyles in the gut, including extracellular and intracellular biofilm populations, planktonic luminal cells, and an intracellular, replicative population. The genetic bases for some of these distinct phenotypes are being elucidated; however, the genetic underpinnings of the adherent and invasive phenotype in general remain elusive. This may be rooted in the fact that AIEC is a context-dependent pathogen, where its virulence and survival determinants are a product of host-guided evolution for which the selective pressures vary between individuals. The broad spectrum of pathological manifestations of CD likely creates different selective pressures in the gut, which may explain, in part, the genetic and phenotypic heterogeneity among clinical isolates of AIEC.

Over the past 25 years since the first description of AIEC, much has been learned about its infection biology, virulence mechanisms, and the genetic and metabolic determinants of *in vivo* fitness. This progress has also helped clarify the role of AIEC in disease activity linked to CD. However, a principal challenge in the field remains at the level of AIEC identification itself. Indeed, substantial variation exists in the phenotypes by which AIEC are defined, and the relative contributions of these phenotypes to disease require more clarification (115). An international panel of experts is meeting in late 2023 to discuss the current classification scheme for AIEC. Based on this discussion, updated recommendations for classifying AIEC may be reported.

In the longitudinal case study of the microbiome dynamics of a CD patient who had high sustained levels of *E. coli*, one dominant strain, ST1, was similar to other AIEC strains in terms of virulence factors, metabolic functions, and phylogeny (35). However, although ST1 adhered to Caco-2 cells, it did not survive to appreciable levels in macrophages, which made its status as AIEC equivocal. It is also possible that in the gut, multiple CD-associated *E. coli* strains with variations in their phenotypes work in concert with one another to influence disease activity. Differentiation of AIEC strains based on their dominant phenotypes may ultimately be helpful in the development of microbiome-targeted therapeutics that are being explored as adjuncts to current IBD treatments.

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