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The invasin IbeA and its role in Adherent-Invasive *Escherichia coli* (AIEC) pathogenesis

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The invasin IbeA and its role in Adherent-Invasive *Escherichia coli* (AIEC) pathogenesis

by

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Dedication

I dedicate this dissertation to my family: My father Javier Cieza and my mother, Maria Cusato, who taught me to always be persistent in order to achieve my life goals, and my sister Ursula and brother Javier. Finally, to all my close friends who were always there to support me when it was needed.

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The invasin IbeA and its role in Adherent-Invasive *Escherichia coli* (AIEC) pathogenesis

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In this study, an Adherent-Invasive *Escherichia coli* (AIEC) Δ*ibeA* mutant was constructed and characterized, and its effect on AIEC adhesion and invasion of intestinal epithelial cells evaluated. The role that this outer membrane protein, IbeA, plays in intra-macrophage survival was also assessed. Compared to the wild-type, AIEC Δ*ibeA* presented reduced invasion to not only enterocytes but also M cells, which correlated with impaired transcytosis through a monolayer of M cells. The observed impairment in invasion was not a consequence of impaired adhesion to intestinal epithelial cells, since this process was not affected. Furthermore, the invasive properties that IbeA confers to AIEC seem to be specific for this pathotype, since complementation of a non-AIEC strain with a plasmid carrying IbeA did not render the recipient strain invasive. Besides intestinal epithelial cells, the other major host cells that are known to be a target for AIEC are macrophages; therefore, the effect of IbeA in intra-macrophage AIEC survival was evaluated, and it was observed that AIEC Δ*ibeA* had reduced intra-macrophage survival when compared to the wild-type, starting as early as 4 hours post infection. Finally, all of these components were integrated by evaluating the contribution of IbeA in an *in vivo* animal inflammation model, and it was found that AIEC Δ*ibeA* was recovered at the same levels observed for the wild-type from mouse intestines. Even though colonization

levels were similar, differences were found at the level of the inflammatory response, reflected in lower pathology scores in the small intestine and the cecum for AIEC Δ*ibeA* when compared to the wild-type, and reduced IFN-γ secretion in the cecum. This study reports, for the first time, the role of the invasin IbeA in the AIEC pathotype, and shows that it plays a relevant role not only in the invasion of intestinal epithelial cells but also in AIEC-associated pathology. This finding opens the possibility of further work evaluating the intracellular mechanisms triggered by IbeA in intestinal epithelial cells in response to AIEC.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Illustrations

List of Abbreviations

AIEC	Adherent-Invasive *Escherichia coli*
APEC	Avian pathogenic *Escherichia coli*
BMEC	Brain microvascular endothelial cells
CD	Crohn's disease
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6
CFU	Colony forming units
DAEC	Diffusely-Adherent *Escherichia coli*
DEC	Diarrheagenic *E. coli*
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
EAEC	Enteroaggregative *Escherichia coli*
ECL	Enhanced chemiluminescence
ECOR	*Escherichia coli* reference collection
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extraintestinal pathogenic *Escherichia coli*
FAE	Follicle associated epithelium
FimH	Type 1 pili minor subunit located at the fimbrial tip
flgC	Flagellar basal body gene
fliC	Flagellin gene encoding the major component of the flagellum
GI	Gastrointestinal
HF/HS	High fat/ High sugar diet
HM605	Adherent-invasive *Escherichia coli* strain
IBD	Inflammatory bowel disease
IbeA	Invasion of brain endothelium protein A
IEC	Intestinal epithelial cells
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-8	Interleukin 8
IpaC	Invasion plasmid antigen C
iutA	Aerobactin receptor gene
LF82	Adherent-invasive *Escherichia coli* prototype strain
Lpf	Long polar fimbriae
M cells	Microfold cells
MEM	Minimum essential media
MLST	Multilocus sequence typing
MOI	Multiplicity of infection
NaH_2PO_4	Monosodium phosphate
Ni-NTA	Nickel-charged resin
NMEC	Neonatal meningitis *Escherichia coli*

NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NRG857c	Adherent-invasive *Escherichia coli* prototype strain
OD	Optical density
OmpA	Outer membrane protein A
OmpC	Outer membrane protein C
ORF	Open reading frame
PI-6	Pathogenicity island 6
PP	Peyer's patches
PSF	Polypirimidine tract-binding protein associated splicing factor
Rac1	Ras-related C3 toxin substrate 1 (Small signaling GTPase protein)
RS218	Neonatal meningitis *E. coli* where IbeA was characterized
ST	Sequence tag
STEC	Shiga toxin-producing *Escherichia coli*
T6SS	Type VI secretion system
TEM	Transmission electron microscopy
TER	Transepithelial electrical resistance
TNF-α	Tumor necrosis factor alpha
UC	Ulcerative colitis
UM146	Adherent-invasive *Escherichia coli* strain
UPEC	Uropathogenic *Escherichia coli*
YfgL	Outer membrane lipoprotein

INTRODUCTION

Chapter 1: Adherent-Invasive *Escherichia coli* (AIEC)

Escherichia coli are the predominant facultative anaerobe present in the human gastrointestinal tract. This Gram-negative bacterium usually coexists with the host for their mutual benefit. At the same time, there are subsets of *E. coli* that have adapted to specific new niches due to their acquisition of virulence attributes. Several subsets of highly adapted *E. coli* have acquired specific combinations of traits resulting in them causing disease in the host. These subsets of highly adapted *E. coli* strains constitute what we call *E. coli* pathotypes and were originally named based on a combination of their clinical spectrum and described virulence factors. Among them, 6 major pathotypes have been described to be associated with intestinal pathologies, enterohemorrhaghic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely-adherent *E. coli* (DAEC) and enterotoxigenic *E. coli* (ETEC), and three with extraintestinal infections or pathologies, namely: uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC) and avian pathogenic *E. coli* (APEC) [1, 2]. Additionally, molecular techniques such as multilocus sequence typing (MLST) are used to establish the relatedness between *E. coli* strains by sequencing a number of housekeeping genes and assigning them a sequence tag (ST). ST can be grouped into five major complexes (A, B1, B2, D and E) based on their similarity, and genetic relatedness of the major pathotypes mentioned above has been established [3, 4].

Adherent-invasive *Escherichia coli* (AIEC) comprises an *E. coli* pathotype that has been associated with Crohn's disease (CD) [5, 6] and primarily belongs in the

phylogenetic group B2 **(Illustration 1)**. The strain LF82 was the first isolate classified as AIEC. This isolate was obtained from a chronic ileal lesion of a patient with CD. The first phenotypical trait described in the AIEC strain LF82 was the capability to invade a subset of intestinal epithelial cells (Intestine-407, HEp-2 and Caco-2 cells); something that later will be described as one of the hallmarks of this newly described pathotype.

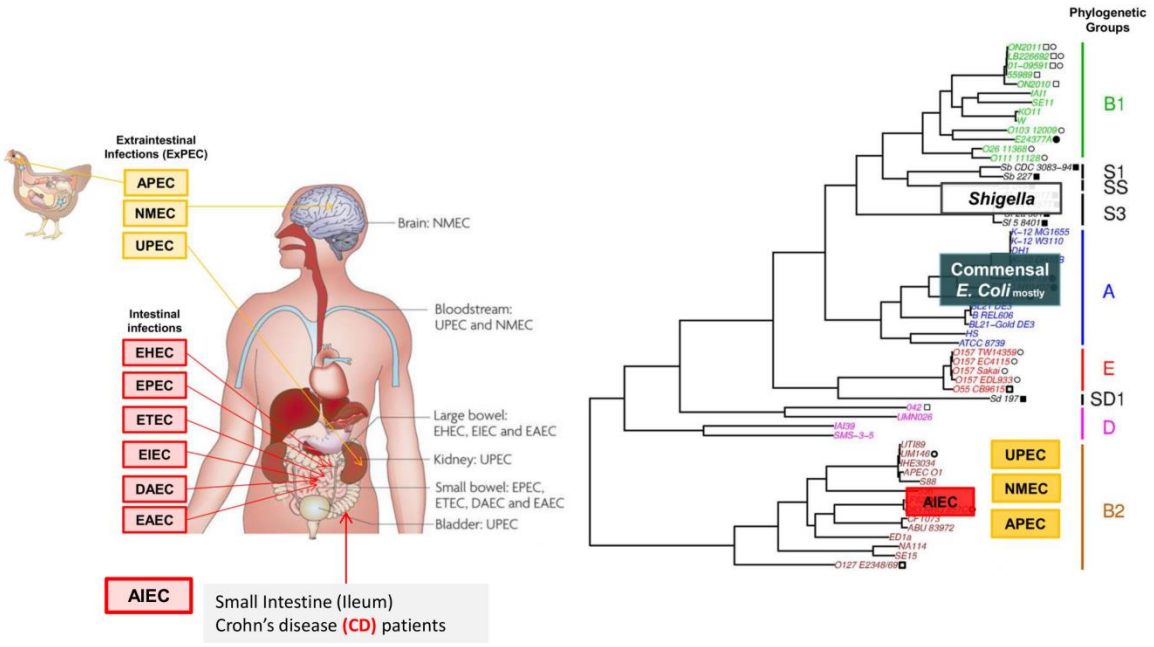


Illustration 1: **AIEC among *E. coli* pathotypes.** AIEC has been associated with intestinal infections such as the counterparts' enterohemorrhaghic (EHEC), Enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroaggregative (EAEC), enteroinvasive (EIEC) and diffusely-adherent (DAEC) *E. coli* as shown in the left side of the panel. However at the genetic level it presents more similarities with strains associated with extraintestinal infections such as uropathogenic (UPEC), neonatal-meningitis (NMEC) and avian pathogenic (APEC) *E. coli*, right side of the panel. Figures adapted from Finlay *et al* 2010 and 2013 [2, 3].

The initial characterization of the strain LF82 by Darfeuille-Michaud *et al* showed that AIEC had significantly higher levels of invasion than did non-pathogenic *E. coli* K12, while the numbers of intracellular bacteria were similar to that observed in enteroinvasive *E. coli* (EIEC) [6]. Interestingly the characterization of AIEC LF82 showed that this strain does not carry several of the known and extensively characterized

virulence factors associated with adhesion and invasion in other enteric bacteria [6]. Such virulence factors include the *eae* gene, which encodes the adhesin intimin in EPEC and EHEC, the *ipaC* gene, which encodes the invasin of *Shigella flexneri* and EIEC, or the *tia* gene encoding the outer membrane associated with ETEC invasion. The absence of these virulence factors makes AIEC a unique subset of isolates [1].

However, it was not until 2002 when AIEC began to be discussed as a newly described *E. coli* pathotype; it was proposed that, based on unique genotypic and phenotypic characteristics, AIEC can colonize and subsequently invade intestinal epithelial cells (IECs) of patients with CD, thus subverting the intestinal barrier. This process would destabilize the intestinal barrier allowing the bacteria to move to deeper tissues within the gastrointestinal tract contributing to an inflammatory process [5, 7].

In the following sections of Chapter 1, current knowledge regarding the genetic characteristics of AIEC as well as its prevalence and proposed role in the pathogenesis of CD will be discussed.

CHAPTER 1.1: AIEC CHARACTERISTICS OF A NEW *E. COLI* PATHOTYPE

Chapter 1.1.1: AIEC genomic characteristics

A relevant problem associated with the study of AIEC has been that until recently, the majority of the studies have been performed in strain LF82 and primarily by one research group [5]. Further understanding of AIEC as a pathotype would require the study of more than one isolate sharing similar genotypic and phenotypic characteristics. Recently, some studies have resulted in the characterization of a number of *E. coli* isolates such as AIEC based on phenotypic similarities with the prototype strain LF82 [8, 9]. However, at the genomic level the similarities with strain LF82 were unknown.

It was not until 2010 that the full genome sequence of the prototype AIEC strain LF82 became available [10]. Since then, a few other *E. coli* isolates have been described to show striking similarities not only at phenotypic level but also at the genomic level with strain LF82, and those include the AIEC strains NRG857c [11], UM146 [12] and HM605 [13].

The chromosome of NRG857c is around 4.7 Mb and encodes for several genes associated with metabolism but also pathogenesis. Several of these genes are encoded in 35 genomic islands, which have been found to be highly conserved in the strains LF82 and NRG857c but absent in other *E. coli* pathotypes, suggesting that they might play a role in defining the phenotype associated with AIEC. Among the potential virulence factors encoded in the chromosome of AIEC, a Type VI secretion system (T6SS) has been reported, present in the strains NRG857c, LF82 and UM146, as well as several fimbrial adhesins and potential mediators of invasion such as a hemaglutinin and the IbeA protein [11]. However, to date the contribution to the AIEC phenotype by most of them has not been proven.

AIEC strains have also been shown to carry an extrachromosomal plasmid; however differences have been found in the plasmids of the two extensively characterized strains NRG857c and LF82. While both of these plasmids encode genes that provide AIEC resistance to a wide range of antimicrobial agents, such as aminoglycosides and β-lactams, they differ in size, and the plasmid of NRG857c, but not of LF82, showed regions of similarity to other *E. coli* pathotypes plasmids [11]. This shows that even though there is a degree of conservation between AIEC isolates, a degree of genetic variability can still be found.

Chapter 1.1.2: AIEC phylogenetic classification

The phylogenetic classification of *E. coli* was established by using an *E. coli* reference collection (ECOR) which was central to the study of *E. coli* diversity, and five major groups were defined (A, B1, B2, D and E). The ECOR collection comprises isolates representative of different geographical regions and hosts, including commensal and pathogenic isolates. This collection, however, was incomplete, since diarrheagenic *E. coli* were under represented, and a collection of diarrheagenic *E. coli* (DEC) was later created to complement the ECOR collection. The five major groups previously identified were also detected in the DEC collection [4].

Upon completion of the genome sequence of the prototype AIEC strain LF82 it was determined that strain LF82 belongs to the phylogenetic group B2 [10]. This meant that strain LF82 shared the highest homology with *E. coli* strains associated with extraintestinal infections (Extraintestinal pathogenic *E. coli*, ExPEC), but at the same time presents enough differences at the genomic level that suggest sufficient divergence to have a variation in the pathology (AIEC presents an intermediate killer phenotype in mice compared to several ExPEC) [10]. AIEC shares several genetic characteristics with ExPEC and also certain phenotypical traits such as the potential to invade epithelial cells and induce inflammatory responses in the host. The characterization of other AIEC strains has solidified the hypothesis that the majority of AIEC isolates fall within the B2 phylogenetic group, as in the case of the strain NRG857c which we have shown also belongs to this phylogenetic group [11].

AIEC are primarily classified based on phenotypic characteristics such as adherence to and invasion of intestinal epithelial cells, lack of known invasive determinants, survival within macrophages and originated from CD patients. However,

close genetic relatedness has been reported among the four AIEC strains that have been fully sequenced and characterized, belonging to the phylogenetic group B2 [10-13]. This does not mean that all AIEC isolates fall within this category, since a minority of isolates have been reported to belong to different phylogenetic groups, suggesting that AIEC can be clonally diverse and belong to more than one phylogenetic group. A larger sample size of strains must be characterized to establish an accurate consensus. It is also interesting to remark that major colonization of *E. coli* from B2 + D phylogenetic groups has been shown in IBD patients differently from what has been observed in healthy subjects [14].

CHAPTER 1.2: PREVALENCE OF AIEC IN CROHN'S DISEASE (CD)

One of the first studies to address the prevalence of AIEC in Crohn's disease (CD) patients was published by Darfeuille-Michaud *et al*. In this study, *E. coli* isolates were obtained from the ileum of 45 patients with CD (23 carrying chronic lesions and 22 patients that had undergone surgical resection of the terminal ileum), and it was found that 21.7% (5 out of 23) of the patients with chronic lesions harbored invasive strains with an AIEC phenotype. The percentage was similar in CD patients that had received surgical treatment and therefore presented early rather than chronic lesions, in which strains with an AIEC phenotype were present in 36.4% of neo-terminal ileal specimens (8 out of 22). In the controls, AIEC isolates were only obtained in 6.2% (1 out of 16) of the patients. It is also important to mention that in that study *E. coli* with the AIEC phenotype were only increased in the ileum of CD patients, since colonic samples showed no differences between CD patients and controls [8]. Other groups have also found an association between AIEC and the intestinal mucosa of CD patients. Elliot *et al* also reported that intracellular *E. coli* were more prevalent in CD patients (90%) than in healthy controls (0%) [15]. Altogether, these studies have shown that the percentage of *E.*

coli with an AIEC phenotype is increased in the intestinal mucosa of CD patients when compared to healthy controls [16].

Interestingly, *E. coli* strains with an AIEC phenotype have not only been found in humans but also in the intestinal tract of cats and dogs with enteritis [17]. In the case of dogs, a previous study had also identified *E. coli* isolates displaying the AIEC phenotype. This subset of strains was primarily associated with granulomatous colitis, a disease with symptoms similar to those of ulcerative colitis (UC) and CD [18]. The inter-species distribution of the AIEC phenotype strengthens the hypothesis that an increased prevalence of AIEC will be observed in association with pathologies comprising inflammation in the gastrointestinal (GI) tract, and this phenomenon could be observed in different hosts.

CHAPTER 1.4: ROLE OF AIEC IN PATHOGENESIS AND ASSOCIATION WITH CD

Inflammatory bowel disease (IBD) includes two conditions, Crohn's disease (CD) and ulcerative Colitis (UC). Both are associated with inflammation in the gastrointestinal tract, but while CD can cause inflammation along the entire gastrointestinal tract of humans, UC is limited to the colon. The etiology of CD and UC is believed to be multifactorial, where genetic predisposition, environmental factors and the microbiota can play a role, leading to disruption of the epithelial barrier's integrity [19, 20]. The disruption of the epithelial barrier can impair different protective mechanisms that this barrier provides, such as alterations in the autophagy process (cytoplasmic degradation system where products are targeted to the lysosome), increased barrier permeability, or a defective mucus layer, resulting in inadequate protection against bacterial adherence and invasion [21]. still being explored whether certain bacteria take advantage of these conditions for proliferation or whether they contribute to the disruption of the epithelial

barrier and inflammation observed in CD [7]. Different bacteria have been associated with CD, such as *Mycobacterium avium paratuberculosis*, *Campylobacter concisus*, *Helicobacter pylori* and *E. coli* [22].

The association of *E. coli* and CD is based on the fact that several reports have shown that in subjects with CD the abundance of *E. coli* with an adherent phenotype is markedly increased. From those *E. coli* with an adherent phenotype from CD patients, an important number of them were found to invade intestinal epithelial cells efficiently, a phenomenon that is not observed in the adherent *E. coli* isolates from healthy subjects [9]. Schippa *et al* [23] also suggested that a subgroup of invasive *E. coli* strains isolated from patients with CD harbored genotypic and phenotypical traits that would benefit their proliferation in an inflamed intestinal habitat. These strains shared a similarity of 80% to the widely characterized AIEC strain LF82. Based on these series of findings and that the AIEC strains LF82 and NRG857c have been obtained from the ileum of CD patients, an association between CD and AIEC has been established.

Martinez-Medina *et al* suggested AIEC as an important candidate contributing to the development of CD in patients with pre-existing genetic susceptibilities and reviewed the pathogenesis mechanism [14]. It was proposed that a pivotal step in the pathogenesis of AIEC is the adhesion via type 1 pili to intestinal epithelial cells overexpressing the surface receptor CEACAM6. Specific variations in the terminal subunit of Type 1 pili are believed to give a competitive advantage to AIEC strains, which will bind to intestinal cells that overexpress CEACAM6 [24]. CEACAM6 (carcinoembryonic antigen-related cell adhesion molecule 6) molecules are exposed on the apical membrane of intestinal epithelial cells and could serve as a platform for bacterial colonization and proliferation [25]. While minimal levels of expression are observed in a healthy intestinal mucosa, CD patients present high levels of CEACAM6 in the ileal mucosa [26]. Even though elevated

levels of CEACAM6 are reported to exist in patients with CD, bacterial infections and pro-inflammatory mediators such as IFN-γ or TNF-α can further increase CEACAM6 expression in the intestinal epithelium, perpetuating a favorable environment for AIEC colonization [27].

Other genetic alterations in human gastrointestinal cells that have also been proposed to contribute to an abnormal colonization of the gastrointestinal tract by AIEC, include NOD2 (nucleotide oligomerization domain-2) [28]. NOD2 can be present in monocytes and Paneth cells of the intestinal epithelium [29], and CD patients have been reported to carry a truncation in one of their leucine-rich repeats (LRR), an association that was exclusive to CD but not to UC [30]. Since NOD2 has been associated with the prevention of harmful inflammatory responses in the small intestine by maintaining the growth of bacterial populations under control [31], it is possible then that in CD patients, the mutations present in NOD2 impair the downstream signaling that enables the intestinal epithelium to appropriately handle intracellular bacteria [29], such as AIEC [32]. Limited experimental evidence is available regarding the role of NOD2 in the intracellular handling of AIEC. One of the available studies showed how this impaired downstream signaling by NOD2 can affect the role of AIEC in the pathogenesis of CD. NOD2 stimulation can drive autophagy via ATG16L1 [33], and ineffective autophagy in the absence of ATG16L1 was not able to restrict intracellular replication of the AIEC strain LF82 [34]. The authors proposed that the mutations in NOD2, could cause the same scenario proposed in their study, where autophagy will be impaired and intracellular replication of AIEC will not be restricted [35].

Finally, diet has been proposed to contribute to the pathogenesis of AIEC, and therefore a possible association with CD. Barnich *et al* [36] reported that under a high fat/high sugar diet (used as a model of western diet) the microbiota of mice changed

leading to alterations in intestinal permeability creating a favorable environment for AIEC proliferation. Under this high fat/high sugar (HF/HS) diet it was found that AIEC caused a significant increase of TNF-α secreted in the colon compared to findings in mice fed with a conventional diet, possibly contributing to the inflammatory state observed in CD. The authors concluded that the western diet, created a favorable environment for AIEC proliferation and therefore a contribution to the etiology of CD. However, it was not explored in this study whether colonization by non-pathogenic bacteria could also be favored under an HF/HS diet [6, 10, 11]. It is possible that the genetic makeup of AIEC allows it to take advantage of this specific HF/HS diet.

While important for the pathogenesis of AIEC, the interactions between type 1 pili with the receptor CEACAM6 or NOD2 mutations in the host are likely not the sole mechanism by which AIEC can establish itself and perpetuate in the host, since these models do not take into account the invasive characteristics of AIEC, or the biology of the microorganism. Furthermore, in cases such as type 1 pili, this fimbriae is not a true invasin [37]. It is important to establish the contribution of other virulence determinants in the pathogenesis model of AIEC, and whether there is a contribution to the pathology observed in CD.

Chapter 2: AIEC-associated virulence factors

In order to understand the interaction of AIEC within a host's gastrointestinal tract, a number of predicted virulence factors associated with adhesion and invasion of the intestinal epithelium, as well as interaction with the underlying immune cells, have been identified within the AIEC genome [10, 11]. The identification of predicted AIEC virulence factors has been possible due to the availability of complete genome sequences of the strains LF82 [10] and NRG857c [11], both of which are broadly accepted prototype AIEC strains and extensively studied. However, our understanding remains incomplete, since only a limited number of these predicted virulence factors identified *in silico* have been further studied and shown to be relevant for host-bacteria interactions *in vitro* and *in vivo*.

Among the host cells that have been described to be relevant for the interaction of AIEC with the host gastrointestinal tract [6], intestinal epithelial cells (IEC), including enterocytes and the specialized microfold cells (M cells), deserve special attention [5, 35]. There are not only morphologic but also functional differences between both cell types. They, together with macrophages, have been reported to be the primary targets of AIEC [5, 14, 28]. Some AIEC virulence factors can also play a role in the colonization of the host, such as the siderophore aerobactin (*iucABCD iutA* operon) [11], which has been reported to play a role in the pathogenesis of diarrheagenic *E. coli* [38, 39] or *arlA* present in the genomic island PI-6, which contributes to the resistance to antimicrobial peptides secreted by Paneth cells [40]; however, they will not be discussed further since the scope of this project is the elucidation of the invasion process of AIEC.

IECs comprise the secretory goblet and Paneth cells, the specialized M cells, and primarily absorptive enterocytes, which border the majority of the intestinal lumen. The

majority of studies associated with bacterial invasion of IEC have been performed with cells that resemble absorptive enterocytes (Caco-2, Hep-2, Intestine-407 and HT-29) [41]; therefore, we will review first the barrier characteristics they provide. While primarily serving as a barrier function in the gastrointestinal tract, they are also indirectly promoting intestinal homeostasis, through the separation of microorganisms and the host immune system, a function that they can also achieve through the secretion of certain antimicrobial peptides [42]. Enterocytes also present highly ordered packed projections of the intestinal epithelium into the lumen, called microvilli which increase the cell surface and facilitate nutrient absorption, while at the same time limit bacterial access to the base of the microvilli and intracellular access [43].

M cells, on the other hand, present a different structure than that of the enterocyte counterpart, with fewer lysosomes, reduced microvilli, and the lack of mucus glycocalix covering the surface [44, 45]. A different pattern of cell surface receptors is also present, which might account for the differences observed in bacterial interaction with enterocytes and M cells. However, the most remarkable characteristic is that, unlike enterocytes, M cells can efficiently mediate transcytosis from the apical surface (lumen) to the basolateral surface (underlying gut-associated lymphoid tissue). This is thought to occur through nonspecific mechanisms such as endocytosis and macropinocytosis; however, receptor-mediated transport has been reported to exist in response to certain bacterial components, a characteristic that enteric bacteria are able to exploit for its benefit [42, 44].

Upon subverting the intestinal epithelial barrier, it has been proposed that AIEC will interact with underlying macrophages, and this interaction of AIEC with macrophages has been reported to play a primordial role in their pathogenesis. Upon infection of macrophages where AIEC can survive, it is reported that it will not trigger

the inflammasome pathway (a multiprotein complex that promotes maturation and secretion of pro-inflammatory cytokines), suggesting that AIEC has evolved a way of escaping the innate immune response from the human mucosa [46].

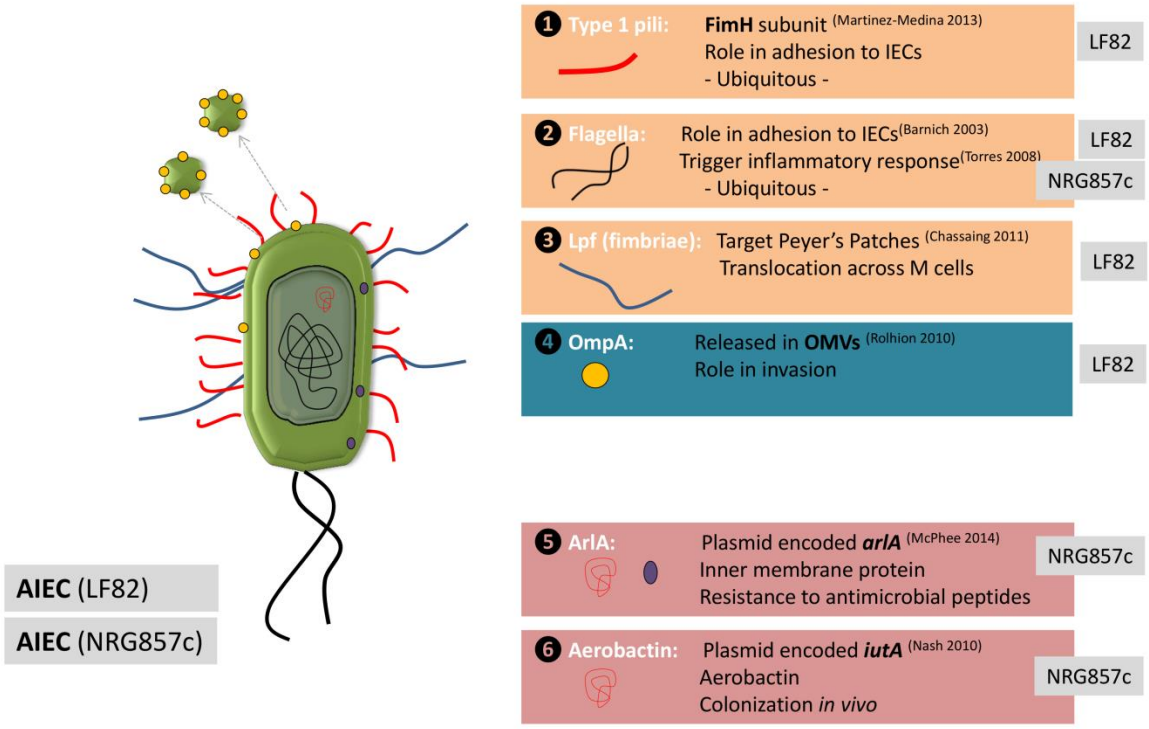


Illustration 2: **AIEC virulence factors.** The virulence factors associated with AIEC adhesion, invasion and colonization of the gastrointestinal tract that are reviewed on this chapter are summarized. In orange boxes the virulence factors associated with adhesion are presented (Type 1 pili, flagella and Lpf); in a blue box, the virulence factor predicted to be associated with invasion (OmpA); and, in purple boxes, the virulence factors associated with colonization or survival within the gastrointestinal tract (aerobactin, ArlA). AIEC virulence factors have been described in the most well-studied strains LF82 and NRG857c.

In this chapter, current knowledge regarding AIEC virulence factors associated with adhesion or invasion of the intestinal epithelium (enterocytes as representative of IEC and M cells as representative of highly specialized IEC), or interaction with the

underlying macrophages are reviewed. In Chapter 2.1 the adhesion factors that mediate close cell attachment of IEC by AIEC are discussed. The majority of these adhesion factors have been studied in the reference strain LF82, where it has been shown that several fimbrial adhesins mediate this process. In Chapter 2.2. I discuss the virulence factors of AIEC associated with invasion. While no true invasin has been described in AIEC, even though invasion of IEC is one of the hallmarks of this pathotype [6, 47], certain virulence factors have been associated with this process. Among them, Outer membrane proteins (Omp) will be described and also the putative invasin IbeA, which potentially can play a role in the invasion process of AIEC. Finally, the virulence factors of AIEC that mediate its interaction with macrophages will be discussed in Chapter 2.3. Altogether the virulence factors associated with the pathogenesis of AIEC are summarized in **Illustration 2**.

CHAPTER 2.1: AIEC ADHESION FACTORS AND INTERACTION WITH INTESTINAL EPITHELIAL CELLS (IEC)

Chapter 2.1.1: Long polar fimbriae (Lpf)

Long polar fimbria is an adherence factor described within some members of the *Enterobacteriaceae* family [48]. Among them, it has been shown to play a role in the adhesion of enterohemorrhaghic *E. coli* (EHEC) O157:H7 to culture cells *in vitro*. The adherence levels of O157:H7 were reduced in 23.4% in the absence of Lpf [49], suggesting these fimbriae may be a component of the adhesion mechanisms of certain *E.coli* pathotypes. Since the aforementioned first report of a functional *lpf* operon in *E.coli*, the presence of an *lpf* operon has been subsequently reported in other *E. coli* pathotypes, such as AIEC [50].

An important role of this adhesin in AIEC is likely, since a functional *lpf* operon has been reported in the prototype AIEC strain LF82. In this study, it was shown that Lpf plays a relevant role in the interaction of AIEC with Peyer's patches (PP), possibly through GP2 receptors that are expressed in the apical membrane of M cells. It is important to note that the interaction of AIEC with M cells through GP2 receptors can also occur through type 1 pili, in a mannose-dependent mechanism. On the contrary, when the interaction of AIEC with M cells is Lpf dependent, this occurs in a mannose-independent mechanism [50]. In the absence of the *lpf* operon, the adherence of AIEC to PP was affected, since a decrease of 6- to 7-fold in associated bacteria to murine PP (*ex vivo* and *in vivo*) was observed when compared to wild-type AIEC. These findings were reproducible to a lesser degree when studied in human isolated PP *ex vivo* [50], suggesting that relevance of Lpf in the interaction with M cells in Peyer's patches is not species-specific. Additionally, it was also observed that the reduced AIEC interaction with PP in the absence of *lpf* resulted in impaired transcytotic capability through M cells, an event that is critical to access the underlying gut-associated immune cells.

The variability of *lpf* can also play a role in the interaction of AIEC with PP. While several *lpf* operons have been observed within the *Enterobacteriaceae* family, and most of them are closely similar genetically, some differences are observed depending on the bacterial species. Upon analysis of the major Lpf structural subunit (*lpfA*) of the AIEC strain LF82 (lpf_{LF82}), it was found to have a similarity of 82 to 86% when compared to *lpfA* from *Salmonella* ($lpf_{Salmonella}$) and enterohemorrhaghic *E. coli* (EHEC). However, these differences did not affect the functionality of Lpf *in vitro*.

Interestingly, in another study, the analysis of a group of AIEC isolates (a group of 6 strains) revealed that not all of them carry an *lpf* operon similar to the one described in the prototype AIEC strain LF82 (lpf_{LF82}). The majority of the isolates presented an *lpf*

operon more similar to the one observed in *Shigella spp.* ($lpf_{Shigella}$) [50]. The most striking difference at the genetic level was in the promoter controlling the expression of the *lpf* operon. Four out of the six strains carried a promoter similar to the one in the prototype strain LF82, while the other two isolates carried multiple substitutions, which were associated with differential expression of *lpf* and, therefore, differences in their ability to interact with PP [51]. These results showed that not all AIEC isolates carry the same *lpf* operon, and this affects the ability of different AIEC isolates to interact with PP.

Finally, environmental conditions can also affect the expression of this adhesin since the presence of a wide subset of bile salts, an important environmental factor within the intestine, was shown to contribute to increased expression of *lpf* in the AIEC strain LF82. Bile salts were the only intestinal environmental factor shown to cause this drastic increase in *lpf* expression, and in this study it was suggested that bile salts favor the specific interaction of AIEC with PP in the intestine [51], possibly increasing the specificity of *lpf* for PP. To sum it up, in AIEC the presence of the fimbrial adhesin Lpf is likely an important factor mediating primarily the interaction with M cells.

Chapter 2.1.2: Type 1 pili

Type 1 pili are the most common adhesive filaments present in *E. coli* and a wide subset of Gram-negative bacteria. While the pilus is composed of one major and three minor subunits, chaperon and usher proteins play an important role in its assembly. The minor subunit, FimH, located at the fimbrial tip is responsible for the interaction with the receptor present in host cells. This interaction can result not only in the invasion of intestinal epithelial cells (IEC) but also in an inflammatory reaction in the host.

In the reference AIEC strain LF82, type 1 pili have been reported to play an important role in adhesion to and invasion of IEC. In an insertional mutagenesis study in the strain LF82, it was found that from the 16 mutants obtained which had an impaired ability to invade IECs, 11 of them carried mutations in the *fim* operon encoding type 1 pili. These strains showed a reduction of 90 to 95% in the numbers of adhered bacteria to IECs compared to wild-type AIEC (100%). This effectively resulted in a reduction of 90 to 95% in the numbers of intracellular bacteria in IEC. It is likely then that the observed impairment in invasion is mainly a product of impaired adherence to IECs. Furthermore similar invasion indexes (% of intracellular bacteria relative to adhered bacteria) were observed when comparing the wild-type AIEC strain LF82 versus the type 1 pili mutant [37].

Interestingly type 1 pili might not contribute equally to adhesion in all AIEC strains, since variability in the terminal subunit FimH within AIEC seems to be a critical determinant of adhesion to the intestinal epithelium. Upon analysis of a collection of 45 AIEC isolates, a clear separation of the strains within two major FimH clades was found, the consensus FimH clade (68.8%) and S70/N78 FimH clade (31.2%). Among them, the AIEC isolates distributed within the S70/N78 FimH clade showed an increased ability to adhere to the intestinal epithelium compared to that of the consensus clade. Not surprisingly, the prototype AIEC strain LF82 belongs to the FimH clade S70/N78 which adheres efficiently to the intestinal epithelium. On the contrary, the AIEC isolates distributed within the consensus, less adherent, FimH clade seemed to share more similarities with non-pathogenic K12 *E. coli* (strain MG1655) which also belongs to this clade, based on FimH subunit similarities [24].

Polymorphisms in the FimH subunit of Type 1 pili seem to not only affect the interaction of AIEC with intestinal epithelial cells *in vitro*, but also the colonization

profile *in vivo*. In a transgenic mouse model expressing the human receptor for type 1 pili (CEACAM6), a 2-log reduction in the number of colonic mucosal-associated bacteria was observed when *fimH* from LF82 (S70/N78 clade) was replaced with *fimH* from K12 *E.coli* MG1655 (consensus clade). The decrease in bacterial colonization correlated with a decrease in the levels of the pro-inflammatory cytokine IL-1β secreted by the colonic mucosa when *fimH* from LF82 was replaced with *fimH* from the consensus clade [24].

This evidence points out again at the difficulties encountered at uniformly classifying *E. coli* strains as AIEC, since within this group of isolates, two distinct genotypic and phenotypic profiles were distinctly observed.

Chapter 2.1.3: Flagella

The role of flagella in the interaction of AIEC with the GI tract has been studied in the strain NRG857c. Adhesion and invasion of non-flagellated AIEC to Caco-2 cells was significantly reduced in this study. However, it is plausible to think that impaired invasion is a by-product of the deficiency in adhesion to Caco-2, as observed with type 1 pili. This impairment of the host-AIEC interaction was directly associated with a reduction in the secretion of the cytokine IL-8 by Caco-2 cells, which in turn resulted in reduced neutrophil recruitment. This was one of the first reports suggesting that by affecting the interaction of the host with AIEC, the inflammatory response could be affected [52]. Subsequently, further studies *in vivo* showed that non-flagellated AIEC behaved more similarly to non-pathogenic *E. coli* since it was incapable of generating an increase of IL-6 and IL-1β in the colon [53, 54].

Further studies of the mechanism by which the absence of flagella might impair adhesion were done on the strain LF82. In these studies it was found that the disruption

of *flgC* by a transposon insertion affected adhesion and reduced invasion. The authors found that the adhesion levels were reduced by 40% as compared to the wild-type, paralleling what was observed in the strain NRG857c where *fliC* was also deleted [52]. In this study it is also possible that an effect over adhesion will subsequently impair invasion. Furthermore the observed reduction in adhesion to IECs (Intestine-407 cell line) mirrors the reduction in invasion. In this study, the absence of flagella also had an indirect effect on adhesion by impairing the expression of another adhesin, type 1 pili [37].

It is important to indicate, however, that flagella is ubiquitous among *E. coli* and not a virulence factor *per se*. Flagella is believed to be more involved in the early stages of interaction with the host, mediating close proximity between the bacteria and the intestinal epithelium. Another factor that obscures the role of flagella in AIEC pathogenesis is that flagellar binding to host cells can vary depending on the serotype, and more than one type of flagellar H antigen has been identified in AIEC even though the widely studied NRG857c and LF82 are O83:H1. Due to all these factors, further clarification of its specificity in AIEC adhesion and invasion is required [55].

CHAPTER 2.2: AIEC INVASION FACTORS AND INTERACTION WITH INTESTINAL EPITHELIAL CELLS (IEC)

Chapter 2.2.1: Outer membrane proteins (Omp)

The role of outer membrane proteins in the invasion process of AIEC was initially described by Rolhion *et al* [56]. The authors found that the deletion of the *yfgL* gene (whose product, YfgL is involved in the synthesis and degradation of peptidoglycan) in the AIEC strain LF82, affected the invasive capability of the strain. The reduction in invasion was a by-product of the impaired ability of the *yfgL* isogenic mutant to produce

outer membrane vesicles (OMVs), since invasion levels of IEC were increased when the *yfgL* mutant was pre-incubated with OMVs from the wild-type AIEC strain. Only OMVs from wild-type AIEC and not from non-pathogenic K-12 *E. coli* caused this increase in invasion. These findings strongly suggested that a component in the AIEC OMVs were facilitating the invasion process [56]. Subsequent studies showed that the components in AIEC OMVs were Outer membrane protein C (OmpC) and Outer membrane protein A (OmpA)[57] [58]. OmpC has been shown to play a role in the invasion of epithelial cells by *Shigella flexneri* but not *Salmonella enterica;* therefore, the authors decided to define whether this protein played a role in the invasion of intestinal epithelial cells by AIEC. It was proposed that the decrease in invasion was more likely attributed to OmpC modulation of a regulatory pathway (i.e., Sigma E) affecting unidentified virulence factors and type 1 pili, rather than a role as an invasin [58]. OmpA on the other hand was found to interact directly with the chaperone Gp96 localized in the apical plasma membrane, thus mediating the internalization of AIEC [57].

Finally, it is worth mentioning that the authors found that AIEC strains with a deletion in *yfgL* also showed a reduction in their motility. However, they proposed that the impairment of motility only affected minimally the invasion process, based on their studies on a flagellum negative mutant of the strain LF82 [37], and, therefore, the differences in invasion of IEC observed in the *yfgL* mutant were not motility related but had to be attributed specifically to a detrimental effect of the invasion process. This is an interesting piece of evidence, which appears to suggest that the invasion process could be independent from bacterial motility.

Chapter 2.2.2: Invasion of brain endothelium protein A (IbeA)

CHAPTER 2.2.2.1: CHARACTERIZATION, STRUCTURE AND SIGNALING BY IBEA

The invasin IbeA (invasion of brain endothelium protein A) was initially described by Kim *et al* in *E. coli* strain RS218 [59]. *E. coli* RS218 is a clinical isolate from the cerebrospinal fluid (CSF) of a newborn infant with meningitis. The strain RS218 can naturally enter the central nervous system (CNS) and cause disease in 50% of the infected animals in a newborn rat model of hematogenous *E. coli* meningitis. However, a single Tn*phoA* transposon insertion mutant of the parental strain RS218 was found to cause meningitis in only 16% of the infected animals, due to impaired access to the CNS. The disrupted gene was proposed to be necessary, if not sufficient, for invasion of brain endothelial cells (BMEC); it was initially named *ibe10* [59], but it was later found to be an 8.2 kDa internal region from IbeA [60].

Further characterization by Kim *et al* revealed that in the strain RS218, the *ibeA* gene coded for a protein with 456 amino acids and molecular mass of 50 kDa [60]. Full recombinant IbeA was expressed in an *in vitro* translation system, and, upon N-terminal sequencing of the recombinant protein (15 N-terminal residues), it was found that the product matched perfectly to the open-reading frame (ORF) described as the *ibeA* gene. Interestingly, the full *ibeA* sequence does not display any significant homology with other known genes present in the GenBank DNA and protein databases, making it a unique gene and virulence determinant.

Additionally, it was shown that IbeA contributed to the pathogenesis of newborn meningitis-associated *E. coli*. Incubation of BMEC monolayers with recombinant IbeA reduced by 80% the invasive capability of the strain RS218. This phenotype was replicated when a non-polar mutation of *ibeA* was generated in the strain RS218 [60].

Further insight into the structure of IbeA was only available when Kim *et al* showed that the protein structure of IbeA comprises an equal percentage of α helix and β-barrels (≈ 30% of each of them), in which most of the β-barrel strands traverse through the outer membrane into the extracellular space. These findings led to the classification of IbeA as an outer membrane protein containing seven transmembrane domains with extended β-sheets and two functional domains [61]. Even though it is widely accepted that IbeA is an outer membrane invasion protein in NMEC strains, Germon *et al* discussed the possibility of IbeA being a cytosolic protein based on the results obtained with PSort analysis which predicts protein sorting signals and localization sites [62]. However, experimental evidence has not supported this claim. Being proposed as an outer membrane protein, two potential receptors in the membrane of brain microvascular endothelial cells (BMEC) were described by Huang *et al* [63, 64].

The first potential IbeA receptor to be identified was vimentin [63]. This protein is a 50-kDa cytoskeleton intermediate filament, which was present in purified BMEC surface proteins and found to bind an internal region of IbeA (between amino acids 281 to 370). This finding was complementary to a previous report by Kim *et al* [59] that proposed that interaction of IbeA with a potential ≈45 kDa protein occurred through an internal IbeA region comprising amino acids 114 to 177. However, Huang *et al* showed that the interaction of IbeA with vimentin was independent from this region [63]. Therefore, further insight into a potential IbeA receptor was required, and Huang *et al* [64] showed that polypirimidine tract-binding, protein-associated splicing factor (PSF) also binds IbeA. It was found that PSF, like vimentin, was present in a BMEC membrane extract and able to bind IbeA. The native conformation of IbeA seemed to be critical for this interaction, since PSF from BMEC membrane extracts was not able to bind denatured IbeA. PSF is a 100-kDa protein which can be localized in the cytosol or the

cell membrane of BMEC [64]. The ≈45 kDa protein interacting with the IbeA region comprising amino acids 114 to 177 has not been identified yet.

The identified IbeA binding proteins, vimentin and PSF, can act in synchrony to facilitate the invasion of BMEC by NMEC. The sole stimulus of recombinant IbeA from RS218, or the bacteria itself, caused increased expression of vimentin and PSF, that resulted in the re-localization of NF-κB (p65) to the nucleus in BMEC. Furthermore, it was found that the physical association between vimentin and PSF was critical for this process since it increased in a time-dependent manner in response to IbeA. Another possible mechanism by which vimentin might mediate *E. coli* intracellular access, it is physical association with autophagic vacuoles. It was proposed that vimentin, as a part of the cytoskeleton network, might actually serve for the transit of autophagic vacuoles (containing the pattern recognition receptor NOD2) to the cellular membrane, where NOD2 will recognize and favor the internalization of the invading pathogen [65].

It is also possible that IbeA can activate the Rho GTPase, Rac1. Brain endothelial cell invasion by NMEC was significantly impaired in the absence of IbeA or upon treatment with Rac1 inhibitors, suggesting that IbeA might mediate the invasion of BMEC upon Rac1 signaling. Whether Rac1 activation occurred downstream of the interaction of IbeA with vimentin and PSF was not explored [66]. To date, these are the only known signaling mechanisms by which IbeA exerts an effect in host cells [67].

CHAPTER 2.2.2.2: GIMA, THE GENETIC LOCUS OF *IBEA*

GimA was originally described in the NMEC strain RS218. This 20.3 kb locus encodes 15 different genes clustered in four different operons (GimA1-4), including the operon where *ibeA* is encoded, the *ibeRAT* operon (GimA4) **(Illustration 3)**. Within the

GimA locus, only *ibeA* and *ibeT* do not show any sequence homology with genes characterized in non-pathogenic *E. coli* K12. GimA also presents characteristics of a pathogenicity island, showing lower G+C content than the rest of the genome (46.2%) and virulence associated genes [68]. However, the locus lacks flanking mobility or insertion elements [69], which have been described as an important characteristic of pathogenicity islands in *Enterobacteriaceae* [70], leading to its classification as a genomic locus instead of a pathogenicity island [69]. Further information regarding the distribution of GimA in the phylogenetic background of *E. coli* populations was only available when Homeier *et al* showed that GimA could be present in three different patterns: the complete locus (GimA+), a 342-bp remnant (GimA remnant) and the absence of any GimA related sequences (GimA-). From a collection of 410 strains including APEC, NMEC, UPEC and isolates from clinically healthy humans and animals, only 16.1% of them were found to be GimA+. The results from this study also implied that the positive strains exclusively belonged to the phylogenetic group B2; however not all members of the phylogenetic group were GimA+ [69]. One caveat of this study is that the collection did not include any intestinal pathogenic *E. coli*, and it would be important to determine the prevalence of *ibeA* in them.

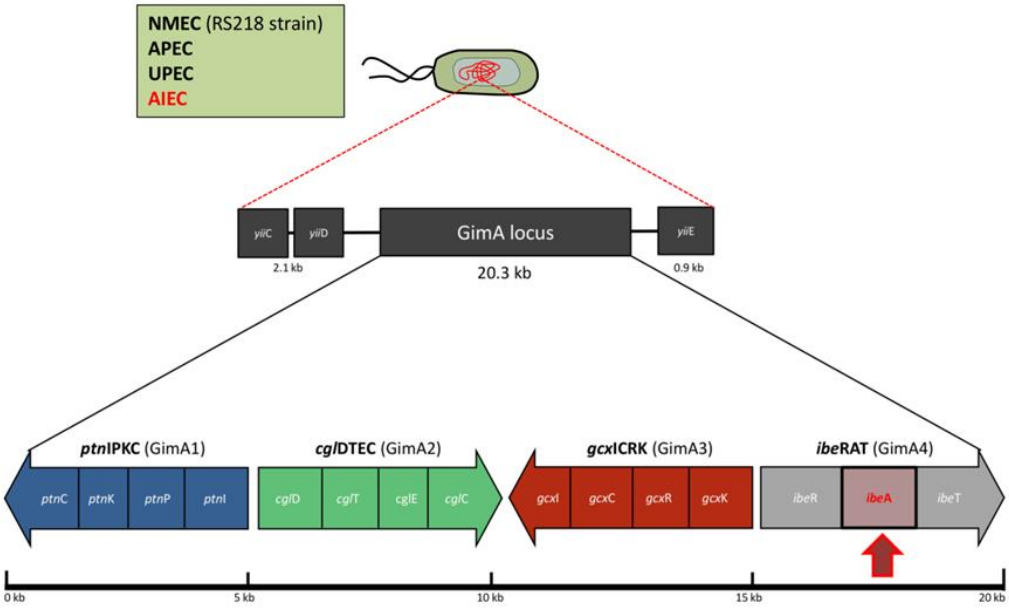


Illustration 3: The GimA genetic locus. The scheme shows the GimA locus, reported to be present in the chromosome of a subset of pathogenic *E. coli*. The four operons (GimA1 – 4) encoded within the locus are represented. GimA1 – 3 are reported to possibly be involved in energy metabolism, while the last operon *ibeRAT* (GimA) has been reported to be involved in bacterial pathogenicity, since it encodes for the invasin IbeA. Figure adapted from Huang et al 2001 [68] and Homeier et al 2010 [69].

Since *ibeA* is encoded within the *ibeRAT* operon (GimA4), further description and discussion will only be centered on *ibeRAT* (GimA4), especially whether *ibeR* and *ibeT* have any contribution to the biology of *ibeA*.

CHAPTER 2.2.2.3: ROLE OF *IBERAT* IN *E. COLI* PATHOGENICITY

IbeR:

The protein sequence homology of *ibeR* initially led to the suggestion that its product works as a regulatory protein belonging to NtrC/NifA family of transcriptional activators [68]. In 2009 that Huang *et al* studied the role of IbeR in an NMEC strain that presents a nonfunctional RpoS (a master controller of bacterial stress response [71]). They proposed that, in this background, IbeR is an RpoS-like regulator based on

functional similarities with RpoS rather than structural homology, which is absent [72]. However, the same report fails to show the importance of IbeR in response to environmental stress or its role as a master regulator, which are important characteristics of RpoS [71], and concludes that IbeR is a regulator with a narrow functional spectrum, primarily involved in the regulation of TnaA, a tryptophanase. TnaA produces an important signaling molecule, indole, which has been associated with the regulation of several aspects critical for the pathogenicity of diarrheagenic *E. coli* [73] and AIEC as shown in our laboratory [74]. By up-regulating the expression of *tnaA*, IbeR increases the availability of indole, which in turn facilitates the pathogenesis of NMEC through more than one mechanism. It is no surprise then that, in the absence of IbeA, a reduction in the invasion of BMEC by NMEC was observed [72]. IbeR, however, does not represent a true invasin, and even though it has been proposed to be the regulatory protein modulating the entire GimA locus [68] in NMEC, no experimental evidence is available.

Recently, Lu *et al* [75] has shown that the regulatory role of IbeR could be dependent on the *E. coli* background in which it is studied, even though the protein showed an identity of 99% in the two pathotypes studied (NMEC [72] and APEC [75]). In this study, the regulatory role of IbeR was studied in an APEC background (with an active RpoS function), instead of an NMEC background, and was found that IbeR could contribute to bacterial resistance to environmental stresses and invasion of chicken embryo fibroblasts. Different from what was found by Huang *et al* [72], they reported that in APEC with active RpoS function, a limited role in the regulation of the GimA locus was present. They found that the gene encoding the invasin *ibeA* was upregulated in the absence of *ibeR*. The authors suggested that in the absence of IbeR, higher expression of *ibeA* will occur in order to compensate for the deleterious effect on invasion caused by the down-regulation of other virulence factors associated with host-bacteria interactions [75]. The study, however, does not conclude if there is a direct regulation by IbeR on

ibeA, and, like the previous study, whether IbeR works as a master regulator of the GimA locus.

It seems however, that the impairment of APEC invasion of chicken embryo fibroblasts by APEC observed in the absence of IbeR is independent from IbeA, since a reduction in invasion occurs even when *ibeA* is upregulated. A double mutant (APEC Δ*ibeR/ibeA*) showed further reduction in invasion of chicken embryo fibroblasts compared to the single mutant (APEC Δ*ibeR*) [75], strengthening the idea that IbeA alone, independent from the presence of IbeR, could cause an effect on invasion.

As shown in these studies, IbeR work as an important regulator of the stress responses and virulence of NMEC and APEC, with different findings observed depending of the bacterial background.

IbeT:

The role of IbeT in the pathogenesis of *E. coli* has been studied in two pathotypes, NMEC [76] and APEC [62]. An isogenic mutant of *ibeT* in the NMEC strain RS218 showed a reduction of 60% in adhesion levels to BMEC when compared to those in the wild-type strain. The invasion levels of BMEC were also affected in the absence of *ibeT*; however, the authors proposed that this is likely a secondary effect caused by the reduction in adhesion, rather than IbeT acting as a true invasin. The authors also proposed that since IbeT predicted structure *in silico* showed three putative extracellular domains, it is likely that IbeT interacts with BMEC surface structures [76]. However, a role of IbeT as an invasin is diminished due to the sequence homology of IbeT with Na (+)/H(+) antiporters rather than other invasins [68]. Finally, this study did not explore whether the absence of IbeT was affecting *ibeA* expression, and, therefore, a potential explanation for

the reduced levels of invasion of BMEC that were observed. The functional relation between IbeT and IbeA still needs further clarification.

In APEC, it was also reported that IbeT can also cause a reduction in invasion, similar to what was observed in NMEC. This group proposed that the reduction in adhesion to chicken embryo fibroblasts observed in the absence of IbeT was likely due to reduced expression of type 1 pili. The authors proposed that IbeT, being a transporter, could contribute to metabolic pathways involved in the expression of type 1 pili, and its absence will cause a metabolic unbalance affecting type 1 pili and, therefore, adhesion [62].

CHAPTER 2.2.2.4: IBEA PRESENCE IN AIEC

As described in the previous sections, all of the studies associated with the role of IbeA in the pathogenicity of *E. coli* have been done in NMEC and APEC. However, AIEC represents a different pathotype [10, 11] and the presence of IbeA in strains classified as AIEC has been described in few studies. Its role as an important invasin for the AIEC pathotype has not been characterized, even though it certainly is a very interesting candidate to study due to the following facts:

i) AIEC lack the classic invasive determinants from *S. flexneri*, EIEC, ETEC and also the *eae* gene that encodes for the EPEC/EHEC adhesin Intimin [5, 6].

ii) The presence of the gene *ibeA* has been reported in fully sequenced AIEC strains LF82 [10] and NRG857c [11] and in a number of *E. coli* isolates described as AIEC [9].

A subsequent study in which 22 AIEC isolates were obtained from four patients with CD showed that 81% of them presented the *ibeA* gene. In the same study, *ibeA* was

non detectable in *E. coli* isolates from healthy control subjects [9]. Another study that has addressed the presence of *ibeA* in a subset of *E. coli* associated with IBD (CD and UC) was published by De la Fuente *et al*. The authors reported 6 isolates with an AIEC phenotype collected from CD patients; however, none of them were reported positive for *ibeA*. Certain discrepancies from what was observed in the literature are observed in this report, since they demonstrate the commensal HS as *ibeA* positive, something never described before, and the AIEC control (strain NRG857c) as *ibeA* negative [77]. It is possible that the primers used in this study failed to detect *ibeA* in several samples. The set of primers used in this study had also been used in a previous study where 9% out of 22 AIEC isolates were reported positive for *ibeA*, while 11% out of 38 non-AIEC isolates were also *ibeA* positive [78]. Further information regarding the region or length of product associated with *ibeA* is not available [79]. It is possible that the detection of *ibeA* in these collections might have resulted in false positives; therefore, it would be extremely interesting to re-evaluate this large collection of IBD-associated isolates to determine the presence of *ibeA*.

To sum it up, there is encouraging evidence of the association between IbeA and the AIEC pathotype. However, the aforementioned studies had some limitations and screening of several AIEC isolates could strengthen the association between AIEC and IbeA.

CHAPTER 2.3: AIEC INTERACTION WITH MACROPHAGES

The interaction of AIEC strain LF82 with macrophages was initially studied by Glasser *et al* [80]. The most relevant findings of this report were that AIEC could efficiently survive within macrophages and was capable of inducing large amounts of TNF-α but not of IL-1β. Further reports showed that the amount of TNF-α secreted was

related to the amount of intracellular bacteria and also with their replication process within macrophages [81]. Interestingly, it was also reported that AIEC uses TNF-α secretion as a mechanism to favor its replication, and this represented a unique mechanism associated with AIEC, since several pathogenic bacteria are actually suppressed by the environment generated by TNF-α [82, 83] . The mechanism behind how TNF-α contributes to AIEC replication in macrophages, might be associated with the ability of TNF-α to induce phagosome maturation (and therefore acidification), contributing to an adequate intra-macrophage niche for AIEC replication [81].

Regarding AIEC's failure to induce IL-1β, this was a very interesting finding since other enteric invasive pathogens, such as *Salmonella* and *Shigella*, do cause the induction of this cytokine and host macrophages to undergo apoptosis [80]. This suggests that even when AIEC manages to survive within macrophages as an invasive pathogen, it does not cause an aggressive activation of an innate immunity in the gut, supporting the hypothesis that AIEC-mediated pathology is due to low persistent activation of gut immunity rather than an initial strong pro-inflammatory response [14, 28]. Jarry *et al* proposed that one of the reasons AIEC does not cause a strong activation of innate immunity, is that it fails to activate the inflammasome (caspases mediating the generated IL-1β and I-18 production in their active forms) [46]. De la Fuente *et al* [77] reinforced the hypothesis that AIEC does not trigger the inflammasome, since AIEC strains from CD patients secreted less IL-1β than the counterpart strains isolated from UC patients. Failure to produce IL-1β might impair bacterial clearance by macrophages. While impaired bacterial clearance might occur due to host genetic alterations in components the innate immune system [29, 31, 33], it is interesting that the bacterial mechanisms contributing to the impairment of this innate immune response have not been described yet.

OBJECTIVES OF THE STUDY

The objective of this study was to characterize an invasin, which might be accountable for the invasive properties associated with AIEC [6]. In the long-term, this will increase our understanding of the interaction of this newly described pathotype, AIEC, with the host, which in turn can clarify the mechanisms by which AIEC contributes to or takes advantage of the pathology observed in Crohn's disease [19, 28]. Furthermore, by establishing the relevance of IbeA as part of the AIEC arsenal of virulence factors, potential diagnosis tools could be developed, whereby this invasin together with other genes associated to AIEC pathogenesis could serve in the identification of AIEC strains in CD patients, without the laborious phenotypical characterization.

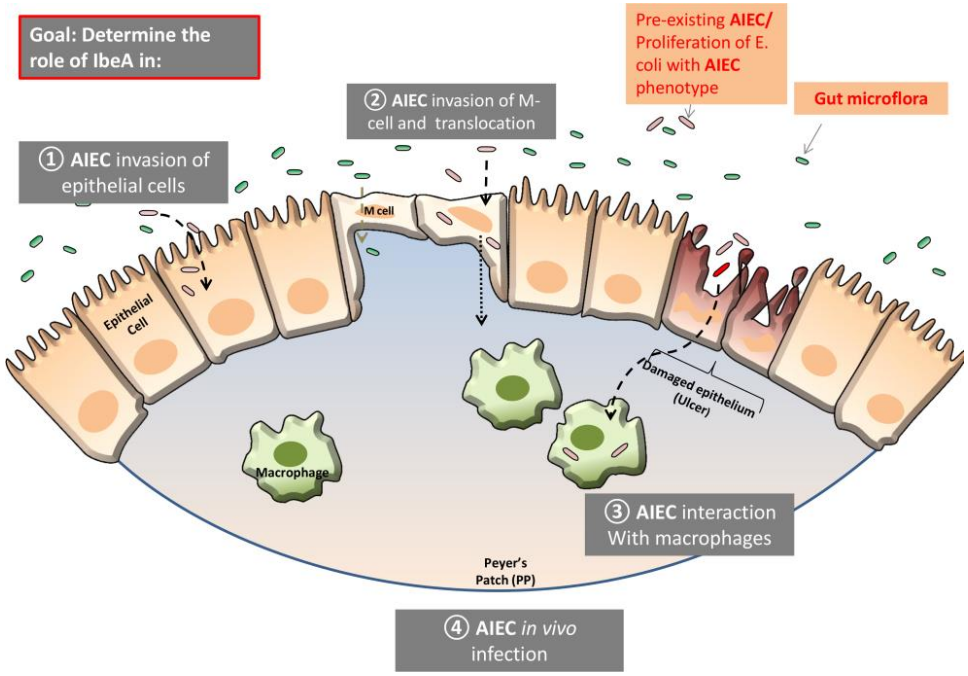


Illustration 4: **Schematic diagram of the objectives of this study.** Role of IbeA in AIEC pathogenesis

The more immediate goals of this project were to describe whether IbeA contributes to the invasion process of IECs by AIEC, something that has not been described before. Additionally, the contribution of IbeA to the interaction of AIEC with M cells was investigated as an additional portal of entry to the underlying gut- associated lymphoid tissue where it could contribute to the persistence of inflammation. Finally the interaction of IbeA with macrophages was assessed, to define whether it affects the survival of AIEC within this cell type, since this is a critical step for AIEC-induced secretion of TNF-α [81] and subsequent inflammation of the host gut. All of these components were integrated by evaluating the contribution of IbeA in an *in vivo* animal inflammation model [84] **(Illustration 4)**.

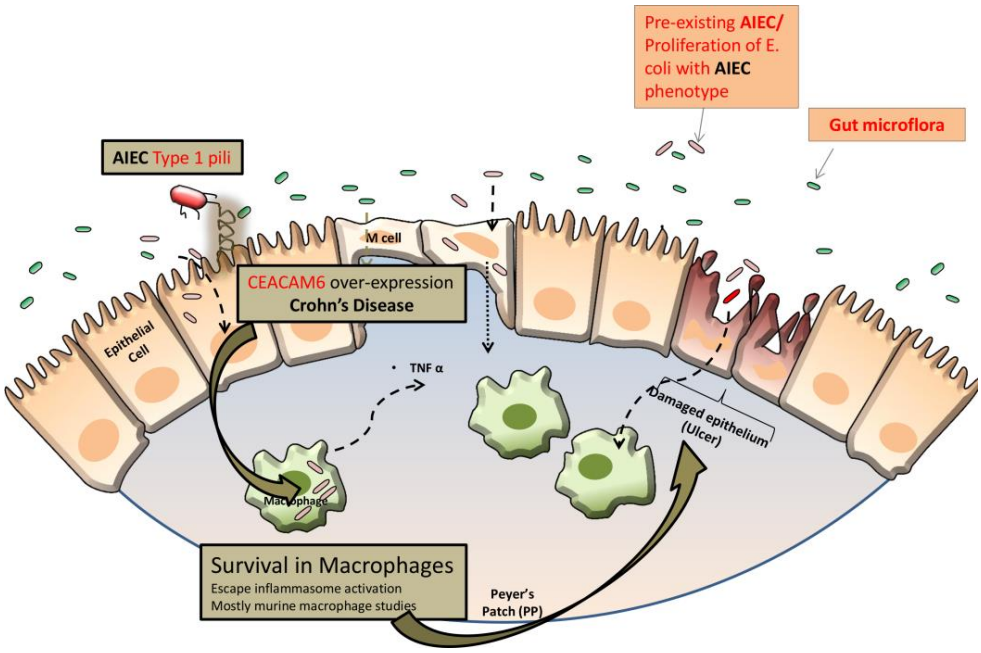


Illustration 5: **Current model of AIEC pathogenies.** Primary interaction of AIEC with epithelial cells occurs through CEACAM6 that is over-expressed under pathological conditions (CD). Upon invasion, AIEC accesses the underlying macrophages where it contributes to the secretion of TNF-α for its survival and perpetuation of inflammation.

By generating this knowledge, another piece of the puzzle will be added to the current model of AIEC pathogenesis. The current pathogenesis model reports primarily that AIEC interaction with the host occurs via type 1 pili – CEACAM6 [26, 27], a widely accepted and relevant hypothesis. This hypothesis, however, it is mainly focused in genetic alterations of a host cell receptor (CEACAM6) and does not take into account other factors present in the AIEC pathotype [10-14] **(Illustration 5).**

MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

Strains and plasmids are listed in **Table 1**. Strains were routinely grown on Luria-Bertani (LB) broth or LB agar at 37^0C. When required, growth media was supplemented with antibiotics at the following concentration: Chloramphenicol (Cm) 30 μg ml^{-1}, Carbenicillin (Cb) 100 μg ml^{-1}, Streptomycin (Sm) 100 μg ml^{-1}, Gentamicin (Gm) 100 μg ml^{-1} and 20 μg ml^{-1}. The AIEC strain NRG857c was used in this study [11, 52]. An *ibeA*$^-$ derivative strain was constructed by disruption of the *ibeA* gene via Lambda Red-mediated gene replacement [85] with the chloramphenicol acetyl transferase gene (*cat*) amplified from the plasmid pKD3 [86]. The primers were IbeA 2-F and IbeA 2-R. The *cat* PCR product flanked by 40 bases upstream and downstream homologous to *ibeA* was subsequently electroporated into Red + Gam-producing AIEC NRG857c (with plasmid pKM201) [74, 85].

For complementation, the *ibeA* gene and putative promoter region were amplified from NRG857c with the following primers RCC20-F and RCC20-R. The primer pair amplified the region coding for *ibeA* from NRG857c while containing restriction sites for *Xma*I and *Bam*HI. The digested product was cloned into the low copy plasmid pACYC177 and the resulting plasmid, pRCC20, was transformed into NRG857cΔ*ibeA*. The plasmid pRCC20 carries a 4.4-kb fragment containing the first two genes of the operon, *ibeR* and *ibeA*, plus the putative promoter region [positions 4662970 to 4666955 of the NRG857c genome, GenBank CP001855 [11]]. The mutant strain complemented with the plasmid pRCC20 was renamed RCC23-1. Another two plasmids (pRCC21 and pRCC22) were generated similarly and contained *ibeA* alone and *ibeRAT* respectively.

These plasmids were transformed into the strain ORN172 for the gain of function studies generating the strains RCC 40-1, RCC 41-1 and RCC 42-1.

Table 1: Bacterial strains, plasmids and primers used in this study

	Relevant characteristics	**Reference or Source**
Strains		
NRG857c	AIEC strain isolated from CD patient Tc^R Ap^R Sm^R Cm^R, O83:H1 serotype	Lab stock [11, 52]
NRG857c (Cu)	AIEC strain isolated from CD patient. Antibiotic sensitive, O83:H1 serotype	[74]
NRG857c Δ*ibeA*	*ibeA* disrupted with *cat* gene amplified from pKD3. Cm^R	This study
RCC 23-1	NRG857c Δ*ibeA* transformed with the plasmid pRCC20 (Cm^R, Ap^R)	This study
ORN172	Δ*fim* strain	Lab stock [87]
RCC 01	Top 10 *E. coli* transformed with the plasmid pRCC01 pBad/Myc-His containing *ibeA* (Cb^R)	This study
RCC 40-1	ORN172 transformed with the plasmid pRCC20 (Cm^R, Ap^R)	This study
RCC 41-1	ORN172 transformed with the plasmid pRCC21 (Cm^R, Ap^R)	This study
RCC 42-1	ORN172 transformed with the plasmid pRCC22 (Cm^R, Ap^R)	This study
MG1666	Prototype K-12 *E. coli* strain. Non pathogenic	Lab stock [88]
HS	Commensal *E. coli* from a human subject	Lab stock [89]
DH5 α	DH5α *E. coli* laboratory strain	Lab stock
Plasmids		
pBAD/Myc-His	Expression vector. Cb^R	Invitrogen
pKM 201	Temperature sensitive red-gam expressing plasmid. Ap^R	[85]
pKD3	Template plasmid containing the *cat* gene template. Cm^R	[86]
pACYC177	3.9 Kb Cloning vector	[90]
pRCC 01	*ibeA* amplified from NRG857c genomic DNA and cloned into pBAD/Myc-His (XhoI and HindIII sites)	This study
pRCC 20	*ibeRA* amplified from NRG857 genomic DNA and cloned into pACYC177 (XmaI and BamHI sites)	This study
pRCC 21	*ibeA* amplified from NRG857 genomic DNA and cloned into	This study

	pACYC177 (XmaI and BamHI sites)	
pRCC 22	*ibeRAT* amplified from NRG857 genomic DNA and cloned into pACYC177 (XmaI and BamHI sites)	This study
Primers		
RCC10-F	TGTACACTCGAGAGAATTTTATCTGGAACCC. Forward primer to amplify *ibeA*. XhoI site. Product length 1362 bp. Cloning into pBAD/Myc-His	This study
RCC10-R	GTGGTCAAGCTTGACTTTTACGCCATTTTGCTG Reverse primer to amplify ibeA. HindIII site. Porduct length 1362 bp. Cloning into pBAD/Myc-His	This study
RCC04-F	ATGGGCCGAAGATGGCATTG. Forward primer to amplify *ibeR*. Product length 1084 bp	This study
RCC04-R	CCCTTGTTGCACGTACTCAC. Reverse primer to amplify *ibeR*	This study
RCC05-F	ATTGCCGCAGCAATGAGTG. Forward primer to amplify *ibeA*. Product length 1075 bp	This study
RCC05-R	GCGGAATCATTACGCCATAAG. Reverse primer to amplify *ibeA*	This study
RCC06-F	GCGTTTTCCTGCATTATTGC. Forward primer to amplify *ibeT*. Product length 1084 bp	This study
RCC06-R	TTCGGGCTAAGACTAACGG. Reverse primer to amplify *ibeT*	This study
IbeA 2-F	TAA ATA TGG AGA CTG GGG GGC GGA TGA AGA AAA TAA AAA CGT GTA GGC TGG AGC TGC TTC. For Lambda red replacement	This study
IbeA 2-R	ATT CAA ATA ATT CGC ATC ACC ATA CTC GGT GAC CGT ACT CAT GGG AAT TAG CCA TGG TCC For Lambda red replacement	This study
RCC20-F	TGTACACCCGGGTATCGACGGCCTGGAAATC. Amplify *ibeA* with XmaI Site. For cloning into pACYC177	This study
RCC20-R	GTGGTCGGATCCACCGATGCCAATAACCAAC. Amplify *ibeA* with BamHI site. For cloning into pACYC177	This study

PCR AND RT-PCR OF *IBEA*

The presence of *ibeA* in the strain NRG857c was confirmed via PCR using the primers RCC05-F and RCC05-R. For confirmation of *ibeA* expression, reverse transcription PCR (RT-PCR) was employed. RNA was extracted from all strains with RNeasy Mini Kit (QIAGEN®) according to manufacturer instructions. Subsequently, 1 µg of RNA was reverse transcribed to cDNA with the Quantitect Reverse Transcription

Kit (QIAGEN®) according to the manufacturer's instructions. The *ibeA* gene was amplified from cDNA by using the primers RCC05-F and RCC05R. Strain MG1655 was the negative control. 16s rRNA was amplified by using 16S universal primers and served as the expression control.

ESTABLISHMENT OF CACO-2 AND M-LIKE CELLS CULTURES

Caco-2 (ATCC® HTB-37™) cells were seeded at a concentration of 5 x 10^5 on the upper side of polystyrene Transwell inserts (3-µm pores, 12-mm filters, CORNING) in 500 µl of complete growth media and cultured until fully differentiated. Caco-2 complete growth media contains Minimum Essential Media (MEM, GIBCO) supplemented with 2 mM Glutamine, 1 mM Sodium pyruvate, 1X non-essential amino acids, penicillin-streptomycin (100 U/ml, 100 µg/ml) and 10% fetal bovine serum (FBS). The basolateral side of the insert was filled with 1.5 ml of complete growth media. Cells were polarized for 14 days. For differentiated, non-polarized Caco-2 cultures, cells were grown until confluency for 4 to 5 days in tissue culture treated 12-well plates (CORNING) and media changed every other day.

For M cell generation, after 14 days post-differentiation (polarization of Caco-2 cells), 5 x 10^5 Raji B lymphocytes were added to the basolateral chamber in 1 ml of complete growth media and maintained for 6 days [91, 92]. The corresponding Caco-2 mono-cultures were used as a control for enterocyte-only monolayers. The integrity of the cell monolayer was measured by transepithelial electrical resistance (TER) [93] before and after the experiments with an STX2 electrode/EVOM^2 epithelial voltohmmeter (World Precision Instruments). Successful establishment of M cell cultures was confirmed by transmission electron microscopy. M cell cultures showed sections with reduced or absent microvilli compared to Caco-2 mono-cultures, consistent with

previous reports [94]. The percentage of Caco-2 cells that acquire M-like cell morphology can range from 15 to 30% of the monolayer [94, 95].

IBEA EXPRESSION, PURIFICATION AND DETECTION

Construction of the expression vector: The sequence corresponding to *ibeA* was amplified from the AIEC strain NRG857c and ligated into a pBAD expression vector. 4.2 kb pBAD/Myc-His (Invitrogen) and the *ibeA* gene (1.4 Kb) amplified with Phusion DNA polymerase (New England Biolabs®) were digested with was digested with XhoI and HindIII. Both fragments were ligated with T4 DNA Ligase and the resulting 5.5 kb plasmid (pRCC 01) transformed into Top10 *E. coli*. The resulting strain RCC 01 was selected to evaluate the expression of recombinant IbeA.

E. coli lysate preparation and recombinant IbeA expression: Expression of recombinant IbeA from RCC 01 was done by inducing the culture (OD_{600} = 0.5) with 0.02% L-arabinose (final concentration) for up to five hours. Samples were collected at 0, 1, 2, 3, 4 and 5 hours post-induction and maximal expression was observed at 3 hours post-induction. In all the time points, cells were harvested at 3000 x *g* for 20 minutes and cell lysates generated upon sonication (20 seconds ON, 40 seconds OFF, 10 pulses at 70% amplitude) under denaturing conditions (100 mM NaH_2PO_4, 10 mM Tris Base, 8M Urea, Roche® protease cocktail inhibitor, pH 8.0).

Recombinant IbeA purification: Purification of IbeA was done under denaturing conditions (as mentioned above) by metal-affinity chromatography (Ni-NTA) according to the manufacturer's instructions (QIAGEN®). Briefly, 2 ml of Ni-NTA agarose (#30210 QIAGEN) were mixed with RCC 01 cell lysate (soluble fraction, 3 hours post-induction) and incubated for 2 hours at 4°C. Ni-NTA agarose incubated with the bacterial lysate was

transferred to a column and washed 4 times (50 mM NaH_2PO_4, 300 mM NaCl, 10-20 mM Imidazole, pH 8.0). The protein was eluted with 250 mM Imidazole, pH 8.0.

Outer membrane protein isolation: For detection of IbeA from AIEC lysates, outer membrane fractions were prepared. Briefly, overnight cultures were pelleted at 7000 x *g* for 15 minutes (OD_{600} of 0.8) and the samples lysed by sonication as mentioned above. Outer membrane fractions were separated by ultracentrifugation at 90000 *x* g for 1 hour (4°C) after removal of cytoplasmic membranes with the solubilization buffer containing 2% (v/v) Triton X-100.

Anti-IbeA antibody: Anti-IbeA antiserum was purchased from Thermo Scientific. The antiserum was generated in rabbits against a 17-amino-acid peptidic region from IbeA (DVFDLTRAEIEGRKQAM, NRG857_21885-285:301). The peptidic region was selected based on the Kyle-Doolittle hydrophobicity test, which measures the hydrophobicity of the region of a protein. Negative scores means less hydrophobic and more hydrophilic regions. Crude serum from immunized rabbits was collected at 60 days post-immunization. To remove unwanted specificities of antibodies (e.g. O, K and H antibodies) the antiserum was absorbed against DH5-α *E. coli* [96].

Western blot and IbeA detection: Recombinant IbeA was detected by western blot with an anti-His antibody at 1/10000 dilution (Invitrogen #46-0693). His-tag immunoreactivity was detected with horseradish peroxidase (HRP) conjugated IgG goat anti-mouse secondary antibody (1/20000 dilution, AbCam #ab97040). For signal detection ECL plus (Thermo Scientific®) was used. IbeA was also detected from AIEC outer membrane preparation. Recombinant IbeA was detected by western blot with an anti-IbeA antibody at 1/4000 dilution (generated for this study). IbeA immunoreactivity

was detected with horseradish peroxidase (HRP) conjugated IgG goat anti-rabbit secondary antibody (1/20000 dilution, Southern Biotech #4050-05).

BACTERIAL ADHESION AND INVASION TO CACO-2 AND M-LIKE CELLS

Cell preparation: For adhesion, Caco-2 and M-like cells were cultivated in 12-well plates. The cells were washed twice with MEM without any supplement prior to infection. Bacterial suspension preparation: The strains were grown on LB media to logarithmic phase (OD_{600} of 1.0) and diluted in MEM to a concentration of 1 x 10^8 CFU/ml. The media from the cell monolayers was then aspirated and 500 µl of bacterial suspension added (~ 5 x 10^7 CFU; MOI of 100) [8, 57] to the apical Transwell compartment. MOI of 1 and 10, were used only in the indicated experiments. The non-polarized Caco-2 cell cultures grown in 12-well plates were infected with 5 x 10^7 CFU in 1 ml of simple MEM. The bacterial suspensions were serially diluted and plated to confirm the bacterial input. Measurement of adhesion: After 3 h, the monolayers were washed four times with PBS and then lysed with 200 µl of 0.1% Triton X-100 and plated on LB agar plates with the corresponding antibiotic. Time points of 10, 60 and 120 min post-infection were evaluated as indicated. Measurement of invasion: A gentamicin protection assay was performed after 3 h of infection, in which the monolayers were washed 2X with PBS and then MEM containing gentamicin (100 µg ml^{-1}) was added to the apical and basolateral chambers of the Transwell system. Monolayers were incubated 1 h and then were washed 2X with PBS. The monolayer was then lysed with 200 µl of 0.1% Triton X-100 and released intracellular bacteria plated for quantification. Readout: The adherent and invasive bacteria were calculated as a percentage of initial inoculum (input) and then adjusted to be expressed as percentage of change compared to the wild-type or as total recovered CFU.

BACTERIAL TRANSCYTOSIS ACROSS M-LIKE CELLS

For the M-like cell transcytosis experiments, the monolayers and bacteria were prepared as described in the previous section. An MOI of 100 (5 x 10^7 CFU) was added to the apical Transwell compartment and after infection, the basolateral media was collected at 0, 1, 2 and 3 h post-infection and bacteria enumerated on LB agar plates. The monolayer integrity was confirmed by measuring the TER. Values above 300 Ω suggest integrity of the monolayer [95]. As a negative control for M cell specific transcytosis, polarized Caco-2 monolayers were used and transcytosis also measured at 0, 1, 2 and 3 h post-infection. Caco-2 monolayers have a reduced transcytotic capability as compared to M cells. All results are expressed as a percentage of the CFU number used for the infection.

BACTERIAL UPTAKE, SURVIVAL AND REPLICATION IN MACROPHAGES

Cell preparation: The human macrophage-like monocyte cell line THP-1 (ATCC TIB-202) was maintained in RPMI 1640 supplemented with 2 mM Glutamine (Gibco 11875-93), 1 mM Sodium pyruvate, 10 mM Hepes, 1X non-essential amino acids, penicillin-streptomycin (100 U/ml, 100 µg/ml) and 10% fetal bovine serum. THP-1 cells were activated to macrophages [97] by using a concentration of 200 nM/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich). THP1 cells (2 x 10^5) were seeded on a 24-well plate and differentiated for 72 h in the presence of 200 nM PMA in 1 ml RPMI 1640. After activation, the media was removed, and cells were left to rest for an additional 72 h [97] prior to infection. Bacterial suspension preparation: The bacterial strains were grown on LB media to logarithmic phase (OD_{600} of 1.0) and diluted in RPMI 1640 to have a concentration of 2 x 10^7 CFU/ml. The cell monolayers were washed twice with PBS prior to receiving 1 ml of the bacterial suspension (2 x 10^7 CFU; MOI of 10) [8, 57]. The bacterial concentration was confirmed and used as the bacterial input. After 1

h of infection (bacterial uptake), monolayers were washed 2X with PBS and incubated with RPMI 1640 plus gentamicin (100 μg ml^{-1}) for 1 h. For later incubation times, the media was replaced with RPMI 1640 plus 20 μg ml^{-1} gentamicin and incubated for 4, 16, 24 and 48 h. Readout: The monolayers were lysed with 200 μl of 0.1% Triton X-100 and the release intracellular bacteria serially diluted and plated on L agar. Survival was expressed as the mean percentage of the number of bacteria recovered at 4, 16, 24 and 48 h compared to that at 1 h post-gentamicin treatment which was defined as 100%. The uptake values were defined at 1 h post gentamicin treatment and expressed as percentage of the infection inoculum.

IN VIVO BACTERIAL INFECTIONS

Mice and treatment: Eight-to-ten-week-old female CD-1 (ICR) mice were purchased from Charles Rivers Laboratories. Animals were housed in a specific pathogen-free barrier under biosafety level 2 conditions. Twenty-four hours before infection, mice were given 25 mg of streptomycin by oral gavage. Infection dose: AIEC strains NRG857c, NRG857Δ*ibeA*, and the K12 strain MG1655 were grown on LB medium overnight at 37^0C. A bacterial suspension of 1 x 10^9 CFU was centrifuged and re-suspended in 400 μl of PBS. Each animal received 1 x 10^9 CFU in 400 μl of PBS via oral gavage, and control groups received sterile PBS. Colonization was determined at 4 days (six mice per group) and persistence at 14 days (seven mice per group) post infection. Readout: After infection, the number of bacteria was monitored in the fecal pellets daily for the first 4 days and subsequently every other until 14 days post infection. Feces were re-suspended in PBS by vortexing and bacteria plated for enumeration. For quantification of bacteria in tissues, sections of ileum (terminal), cecum and colon were collected at 4 and 14 days post infection in 15-ml tubes containing PBS and homogenized by using the Covidien Precision Disposable Tissue Grinder Systems. The re-suspended

feces and tissue homogenates were then serially diluted and plated on MacConkey agar containing streptomycin (100 μg ml^{-1}). After overnight incubation at 37^0C, colonies were counted and expressed as either CFU per gram of feces or CFU per organ.

CYTOKINE QUANTIFICATION

Colon and cecum were removed and fecal contents collected. To measure the local IFN response, tissue sections (3 small punches) were then incubated in 1 ml of complete RPMI (10% fetal bovine serum, 1% L-glutamine and 50 μg ml^{-1} of gentamicin) for 18 h. IFN-γ and TNF-α levels were determined by using the respective ELISA Ready-Set-Go kits from eBioscience according to the manufacturer's instructions.

HISTOPATHOLOGICAL EVALUATION OF MICE TISSUES

Sections of mouse small intestine (terminal), cecum and colon were excised at 14 days post infection and washed with PBS. The sections were fixed in buffered 10% formalin, paraffin-embedded, sectioned into 5-μm slices and then stained with hematoxylin and eosin at the Histopathology Core at UTMB. Three sections from the same tissue (small intestine, cecum and colon) from each animal were stained and scored according to the histopathological scoring criteria used previously by Small *et al* [98]. In these criteria, the lumen, surface of the epithelium, mucosa and submucosa are scored separately. The histopathological scoring was performed blindly by Dr. Elena Sbrana (UTMB).

ELECTRON MICROSCOPY

Transwell inserts containing Caco-2 cells or M-like cells were washed gently with PBS, and fixed in a mixture of 2.5% formaldehyde, 0.1% glutaraldehyde, 0.03% trinitrophenol, and 0.03% $CaCl_2$ in 0.05 M cacodylate buffer (pH 7.2). Samples were processed further by post-fixing in 1% OsO_4, stained *en bloc* in 1% uranyl acetate (in 0.1 M maleate buffer, pH 5.2), and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Specimens were examined in a Philips 201 electron microscope.

STATISTICAL ANALYSIS

One-way analysis of variance followed by Tukey's post-test analysis was performed when comparing more than two groups and multiplicity adjusted p values reported. Kruskal-Wallis One-way ANOVA followed by Dunn's multiple comparisons test was used for comparisons in the animal experiments to the variance of the populations. A 95% confidence interval was used for most of the analysis. For the macrophage survival assay and transcytosis experiments, a two-way ANOVA or two-way ANOVA repeated measures were used with a Bonferroni post-test analysis. All analysis was performed using GraphPad Prism 6.0 (GraphPad Software Inc.) A p-value of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

Chapter 3: The invasin IbeA has an effect on the interaction of AIEC with intestinal epithelial cells (IEC) and macrophages.

INTRODUCTION

In the following section, the results related with the role of IbeA in the invasion process of AIEC will be summarized. IECs and M-cells were used as models for adhesion and invasion, as well as transcytosis through M-cell monolayers. Furthermore the role of IbeA in the interaction between AIEC and macrophages is documented. As mentioned in the previous sections, invasion is one of the hallmarks of AIEC [5, 6], a process that is still not fully understood since AIEC lacks several of the described invasins associated with diarrheagenic *E. coli* and *Shigella* [10, 11]. This is an important process that can allow AIEC to breach the intestinal epithelial barrier and access the underlying gut-associated lymphoid tissue, where, upon interaction with macrophages, AIEC can contribute to intestinal inflammation [14, 23]. In this section, we describe the construction and characterization of a Δ*ibeA* mutant in the AIEC strain NRG857c and the effect this has on the adhesion and invasion of the intestinal epithelium as well as macrophages. The information generated is an important new piece of the puzzle to understand how AIEC establishes the interaction with host cells and possible association with inflammation.

RESULTS

Chapter 3.1: Mutant construction and complementation

A previously described method by Datsenko *et al* for disrupting *E. coli* chromosomal genes was used to generate an *ibeA* mutant in the AIEC strain NRG857c [86]. By this method a disruption of the *ibeA* gene was generated by inserting a selectable antibiotic resistance marker within the *ibeA* sequence. The selectable antibiotic resistance marker inserted was the chloramphenicol acetyl transferase (*cat*) gene amplified from the plasmid pKD3. The presence of the *cat* gene was subsequently confirmed by PCR. In order to restore the wild-type phenotype and confirm that the phenotypic changes observed in AIEC occurred because of the absence of *ibeA*, the complemented strain RCC23-1, which carries the promoter region of the operon *ibeRAT* plus the *ibeA* gene was generated.

The generation of this Δ*ibeA* derivative strain was confirmed at genetic and transcriptional levels. The band corresponding to *ibeA* showed a shift in its molecular size due to the *cat* gene insertion (**Figure 1A**), while at transcriptional level the band corresponding to *ibeA* was absent in AIEC Δ*ibeA* confirming the effective inactivation of the gene and restored in the complemented strain (**Figure 1B**). The intensity of the *ibeA* band appeared stronger in the complemented strain when compared to that of the wild-type strain, a finding commonly reported when plasmids are used for genetic complementation, since even low copy number plasmids can exceed the chromosomal number [99]. As negative controls, a laboratory-adapted *E. coli* K-12 strain was used (MG1655) and also a human commensal *E. coli* isolate, HS [100]. Both strains have been shown not to be associated with human pathology, and the GimA island (which contains *ibeA*) is absent in both of them **(Figure 1A)**. It can be observed that the inactivation of

ibeA or complementation did not have an effect on the growth of the newly generated strain when compared to that of the wild-type AIEC strain NRG857c **(Figure 1C)**.

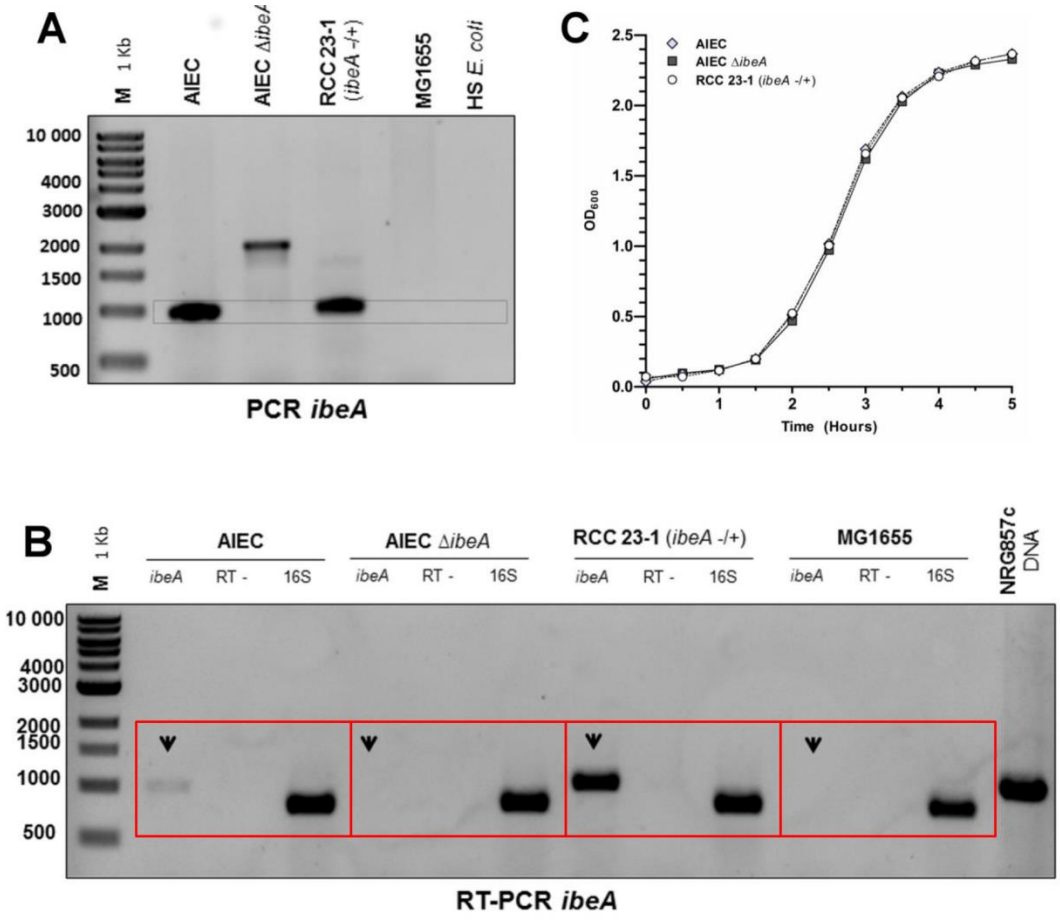


Figure 1. **Deletion of *ibeA* at genomic and transcriptional levels. (A)** Genomic DNA from AIEC strain NRG857c, NRG857c Δ*ibeA* (AIEC Δ*ibeA*) and the complemented RCC23-1 strain (Δ*ibeA*/*ibeA*+) were used to test for the presence of *ibeA*. As negative controls the strains MG1655 and *E. coli* HS were used. **(B)** RNA was also extracted and RT-PCR performed on the same strains. 16S ribosomal RNA was used as an internal expression control. The absence of *ibeA* can be observed in NRG857c Δ*ibeA*, while a transcript is observed from the wild-type strain. **(C)** The growth of the strains was monitored in LB broth up to 5 hours and no significant differences were found.

We also confirmed that the disruption of the *ibeA* gene did not affect the other two members of the operon (*ibeR* and *ibeT*). As reviewed in Chapter 2.2.1.3, *ibeR* and *ibeT*

can have an effect on the pathogenesis of *E. coli*, so it was necessary to confirm that a polar effect due to the disruption of *ibeA* was absent. As observed in **Figure 2**, the transcripts corresponding to *ibeR* (upstream of *ibeA*) and *ibeT* (downstream if *ibeA*) were present in NRG857c Δ*ibeA* (AIEC Δ*ibeA*). The control strain MG1655 was also negative to *ibeR* and *ibeT* as expected.

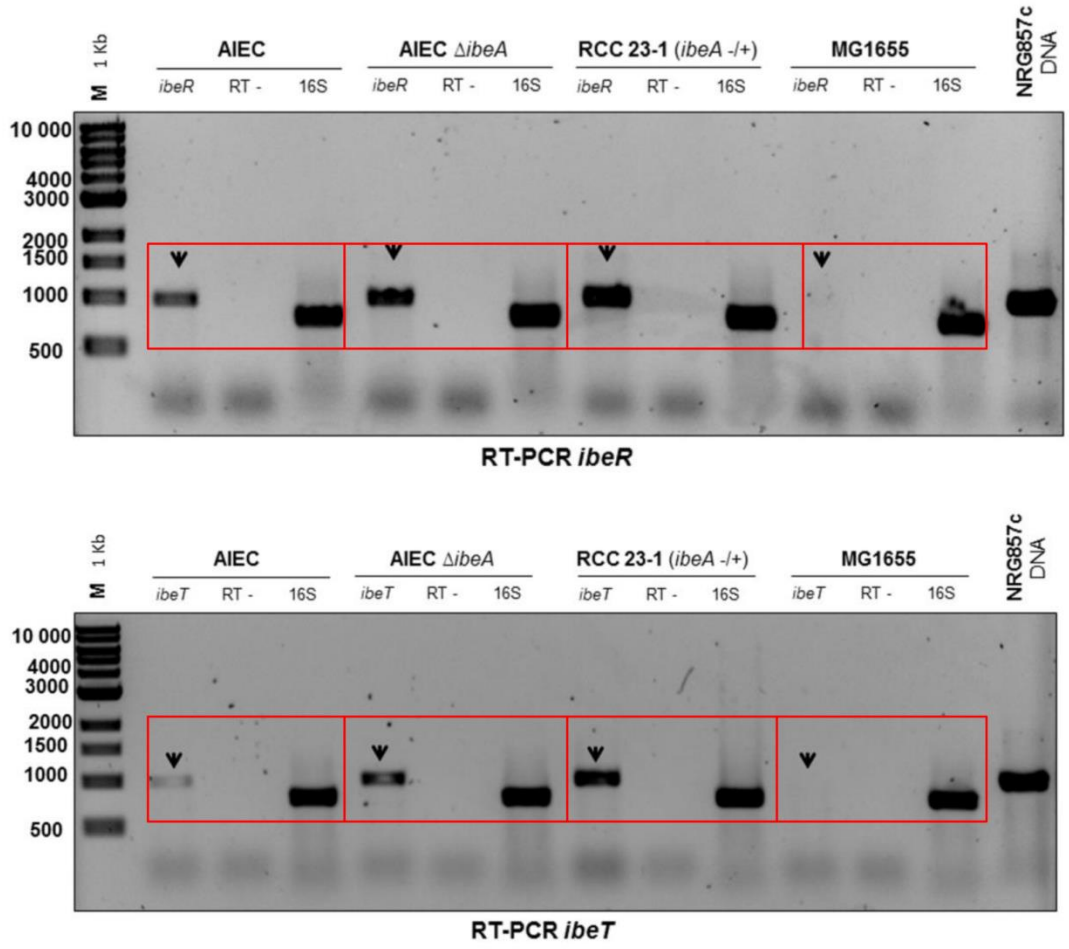
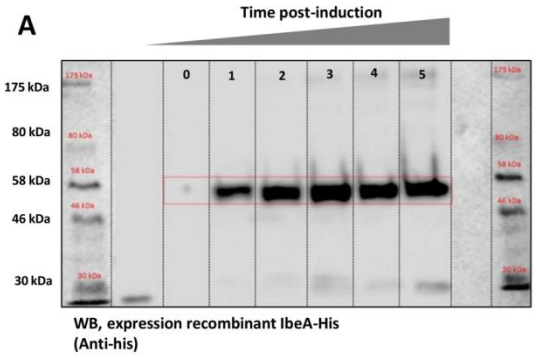
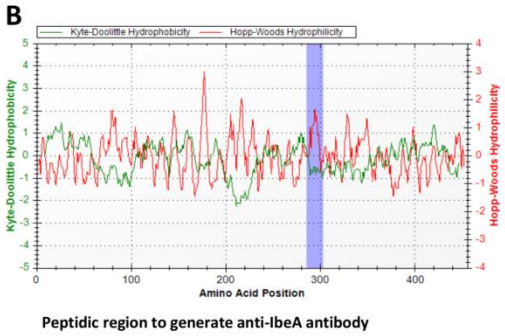


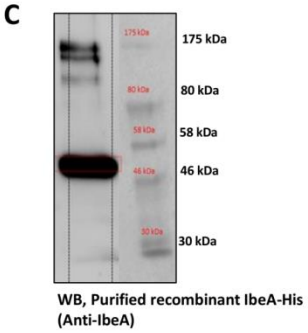
Figure 2. **Effect of *ibeA* deletion in the other members of the *ibeRAT* operon.** RNA was extracted and the effect on the two other members of the operon was evaluated by RT-PCR for *ibeR* (top panel) and *ibeT* (bottom panel). The nonpathogenic *E. coli* K12 strain MG1655 was used as a negative control. The AIEC strain used for this experiment was NRG857c. 16S ribosomal RNA was used as internal expression control.

Chapter 3.2: Generation of recombinant IbeA and purification

In order to increase protein expression, *ibeA* was cloned in the expression vector pBAD/Myc-His. The recombinant protein generated had a molecular weight of around 50 kDa as already predicted by Kim et al [60, 61]. Expression of IbeA was induced in strain RCC 01 with L-arabinose. The induced protein His-tagged IbeA was efficiently recognized by an anti-His tag antibody **(Figure 3A)**. Subsequently an anti-IbeA antibody was designed targeting a peptidic region of 17 amino acids starting at position 285 of the 456 amino-acid sequence **(Figure 3B)**. The peptidic region was selected based on the Kyle-Doolittle hydrophobicity test [101]. A hydrophilic region was selected, because those regions are usually located in the outside of the membrane, and exposed for the detection of the antibody.







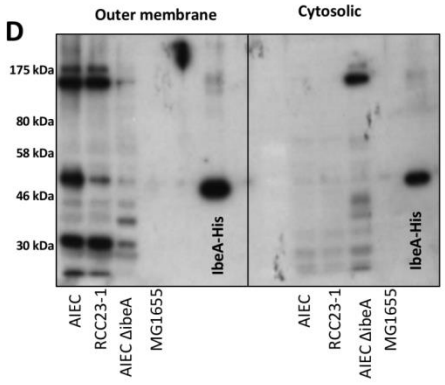


Figure 3. Generation of recombinant IbeA and detection. The *ibeA* gene was cloned into the expression plasmid pBAD/Myc-His and expressed in TOP10 cells. **(A)** Expression of IbeA throughout a time course of 5 hours of induction with 0.02% L-arabinose and detected with an anti-His tag antibody. **(B)** Peptidic region chosen to design the anti-IbeA antibody. **(C)** Detection of the recombinant IbeA (His-tagged) by the anti-IbeA antibody. **(D)** Detection of IbeA in outer membrane and cytosolic fractions of AIEC. All gels were 7% SDS-PAGE. A broad range protein marker ladder was used (7-175 kDa).

The anti-IbeA antibody generated was able to detect the recombinant IbeA (His-tagged) **(Figure 3C)** and subsequently it was also tested against outer membrane lysates from AIEC wild-type, the *ibeA* mutant and the complement **(Figure 3D)**. No protein was detected in the cytosolic fraction, compared to the outer membrane fraction, even though equal amounts of protein were used for both fractions. The expression levels in the complement strain, did not reach levels of the wild-type in this experiment. The presence of IbeA in the outer membrane fraction contradicts the prediction by Germon [62] suggesting that IbeA might be a cytosolic protein, and fits the results obtained by Kim *et al* [60, 61].

Chapter 3.3: Role of IbeA in adhesion and invasion of Caco-2 cells

After confirming the deletion of *ibeA* at a genetic level, we wanted to determine whether a phenotypic was observed. Upon infection of non-polarized (differentiated) Caco-2 cells and infection with the AIEC NRG857c, *ibeA* mutant and complemented strains, the invasion levels in NRG857cΔ*ibeA* were reduced to 64.5 ± 3.1% compared to the levels observed in the wild-type strain (p <0.0001) at an MOI of 100. Complementation with the *ibeA*-expressing plasmid partially restored the invasion levels, to 87% of the wt (p = 0.029) **(Figure 4B)**. When measuring the adhesion levels, we found a slight reduction to 81.8 ± 5.8% (p = 0.11) **(Figure 4A)**. These results suggested that

IbeA played a major role in the invasion of differentiated intestinal epithelial cells by AIEC, while adhesion was not significantly affected at 3 h post-infection.

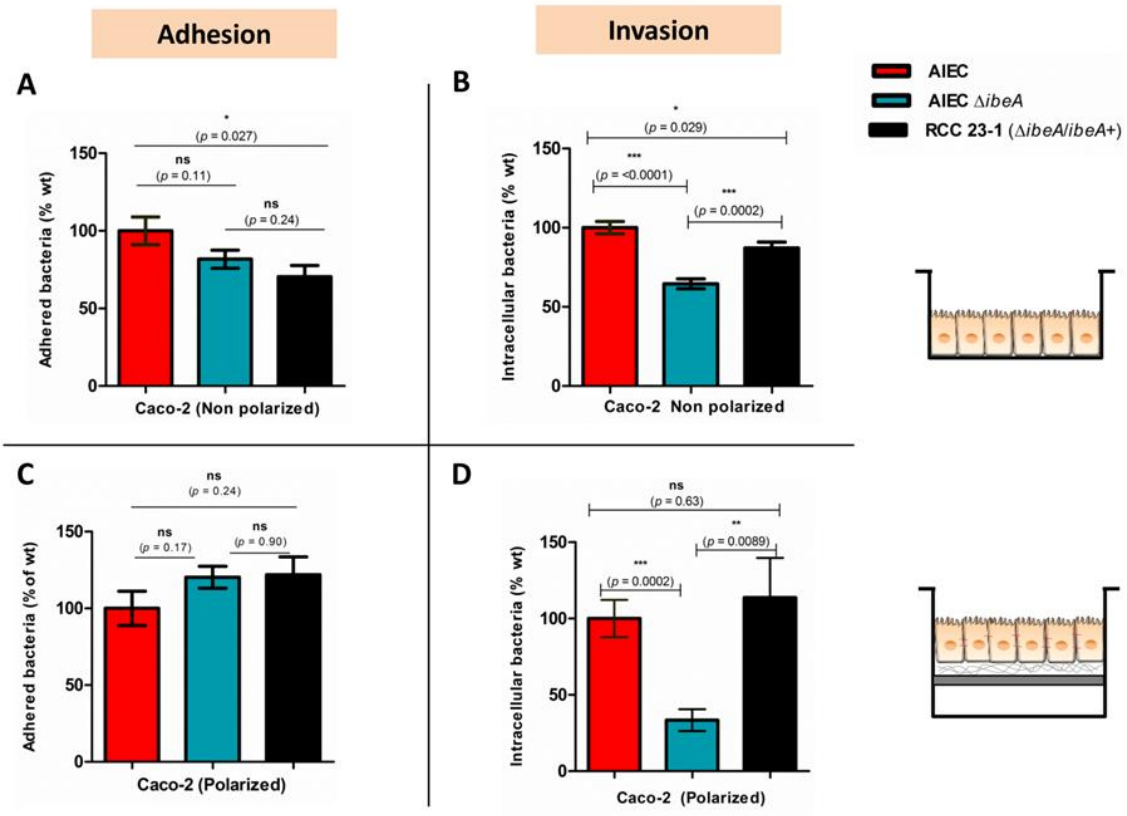


Figure 4. Effect of IbeA on adhesion and invasion of AIEC of Caco-2 cells. AIEC adhesion (**A** and **C**) and invasion (**B** and **D**) were measured in non-polarized (**A** and **B**) and 14-days polarized (**C** and **D**) Caco-2 cells. The top panel represents differentiated Caco-2 cells and the bottom panel differentiated and polarized Caco-2 cells. The wt AIEC strain NRG857c is represented in red bars, NRG857cΔ*ibeA* (AIECΔ*ibeA*) is in blue bars, while the complemented RCC 23-1 strain (Δ*ibeA*/*ibeA*+) is in black bars. For all the experiments an MOI of 100 was used and bacteria were recovered and quantified at 3 h post infection. Data are expressed as the means ± S.E. from three independent experiments (n = 9). Not significant (ns) if $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$ for comparisons between groups (One-way ANOVA followed by Tukey's multiple comparisons was used).

When the same strains were tested in polarized (differentiated and polarized) Caco-2 cells, we found the differences even more prominent. The invasion levels of the strain NRG857cΔ*ibeA* were reduced to 33.4 ± 7.1% of the wild-type levels ($p = 0.0002$),

and the complemented strain restored the invasion to 113.7 ± 26% of the wild-type (p = 0.63). The adhesion levels were consistent with those observed in non-polarized (differentiated) Caco-2 cells, and they were not significantly different, 120.3 ± 7.1% of wt levels (p = 0.17) **(Figure 4C, D)**. Overall, these results confirmed the role of IbeA in the AIEC invasion process. The data showing that IbeA participated in AIEC invasion also demonstrated that differences were not associated with damage to the cell monolayer, since the transepithelial electrical resistance (TER) was not significantly affected between the different groups tested (The mean TER for Caco-2 polarized monolayers is ≈350 Ω).

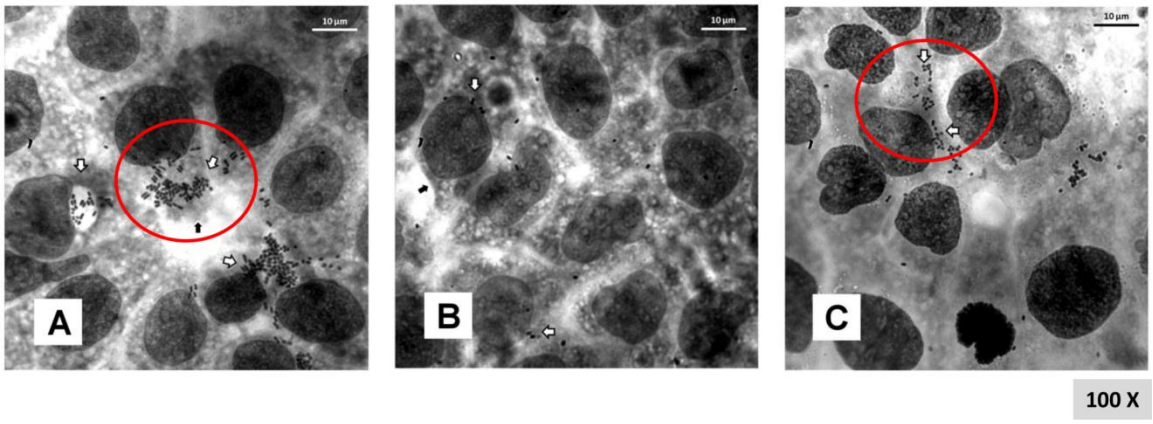


Figure 5. Visual examination of invasion of AIEC on Caco-2 cells. Upon infection for 3 h, monolayers were treated with gentamicin and fixed with methanol stained with Giemsa. Monolayers were visualized at 100X magnification. Monolayers were infected with the wild-type AIEC strain NRG857c **(A),** NRG857cΔ*ibeA* (**B**) or the complemented RCC 23-1 strain (Δ*ibeA*/*ibeA*+) **(C).** White arrows mark sections of the monolayer where bacterial aggregates were present.

Caco-2 cells are a well-established and accepted *in vitro* model for human intestinal epithelial cells, and growing conditions can affect the functional properties of differentiated Caco-2 monolayers [102]. After Caco-2 monolayers reach confluency,

around 5 days post seeding, cells are reach differentiation, however subsequent polarization of the monolayers requires more than 14 days to be completed [103].

It is important to point out that differentiation and polarization of Caco-2 cells are related, but different, processes, since after polarization the apical localization of microvilli and presence of tight junctions are observed (structural polarity) [104]. Furthermore, polarization of Caco-2 cells has been reported to effectively affect the bacteria-epithelial cell interaction, making the monolayer surface less accessible and the barrier more restrictive [105], while also presenting different distributions of cell surface receptors. A clear example of this has been reported in the case of *Listeria monocytogenes* where after 14 days of polarization, the percentage of invasion of Caco-2 cells diminished from 1% to ≈0.1% [106].

Table 2. Bacterial levels on Caco-2 cells and M cells during adhesion and invasion experiments.

	Adhesion					
	NRG857c (wild-type)		**NRG857cΔ*ibeA***		**RCC23-1** (Δ*ibeA*/*ibeA*+)	
	CFU recovered	**wt**	**CFU recovered**	**% of wt**	**CFU recovered**	**% of wt**
Caco-2 (non polarized)	**1.39E+06** ± 1.48E+05	**100%** ± 8.1	**1.19E+06** ± 9.34E+04^{ns}	**81.8%** ± 5.8^{ns}	**9.67E+05** ± 8.19E+04*	**70.3%** ± 7.3*
Caco-2 (polarized)	**2.99E+06** ± 3.35E+05	**100%** ± 11.24	**3.45E+06** ± 2.05E+05^{ns}	**120.3%** ± 7.1^{ns}	**3.75E+06** ± 3.40E+05^{ns}	**121.9%** ± 11.6^{ns}
	Invasion					
	NRG857c (wild-type)		**NRG857cΔ*ibeA***		**RCC23-1** (Δ*ibeA*/*ibeA*+)	
	CFU recovered	**wt**	**CFU recovered**	**% of wt**	**CFU recovered**	**% of wt**
Caco-2 (non polarized)	**1.70E+04** ± 1.44E+03	**100%** ± 3.9	**8.95E+03** ± 8.16E+02***	**64.5%** ± 3.1***	**1.54E+04** ± 9.21E+02*	**87.1%** ± 3.8*
Caco-2	**2.55E+03**	**100%** ±	**9.78E+02** ±	**33.42%**	**2.31E+03** ±	**113.7%**

(polarized)	± 3.99E+02	12.19	2.57E+02***	± 7.1***	5.12E+02^{ns}	± 26^{ns}
	Values represent the mean ± SE at each time point					
	* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ denote statistical significance when compared to NRG857c					
	The CFU recovered for the wild-type strain NRG857c were set as 100%, in each experiment. MOI of 100 (5 x 10^7 CFU)					

The effect of polarization on Caco-2 cells (differentiated and polarized) might explain why, when measuring the invasion levels of the wild-type NRG857c strain, it was observed that the CFU recovered in polarized Caco-2 cells were 2.55 x 10^3 CFU, while in non-polarized Caco-2 cells (differentiated) the values were one log higher, 1.70 x 10^4 CFU. Interestingly, this difference in CFU levels were not observed when adhesion was measured, 1.39 x 10^6 CFU in non-polarized versus 2.99 x 10^6 CFU in polarized Caco-2 cells, supporting the idea that polarization had an impact on restricting the invasion event. The values are presented in **Table 2**.

Finally, non-quantitative visual representation of the changes in AIEC invasion associated with IbeA can be observed in **Figure 5**. In Caco-2 monolayers (non-polarized treated with gentamicin, larger accumulates of intracellular bacteria can be observed within the cytosol of the cell infected with the wild-type and complemented strains in contrast to the *ibeA* mutant.

Chapter 3.4: Role of IbeA on the kinetics of adhesion and invasion of Caco-2 cells

Upon determining that the absence of IbeA affected AIEC invasion of IEC, it was important to find whether the impairment of invasion was reproducible at different time points. The role of IbeA during adherence and invasion assays was evaluated by comparing the wild-type strain NRG857c, NRG857cΔ*ibeA* and the complemented strain RCC23-1 (Δ*ibeA*/*ibeA*+) using non-polarized Caco-2 cells. The adhesion and invasion of

all the aforementioned strains was studied at the following time points: 10, 60, 120, and 180 minutes. It was found that deletion of *ibeA* in NRG857c caused a transient phenotype with significantly increased adherence at early time points but that these diminished thereafter. The amount of adhered bacteria as a percentage of the inoculated bacteria was similar for the wild-type and NRG857cΔ*ibeA* upon 3 hours of infection **(Figure 6A, B)**.

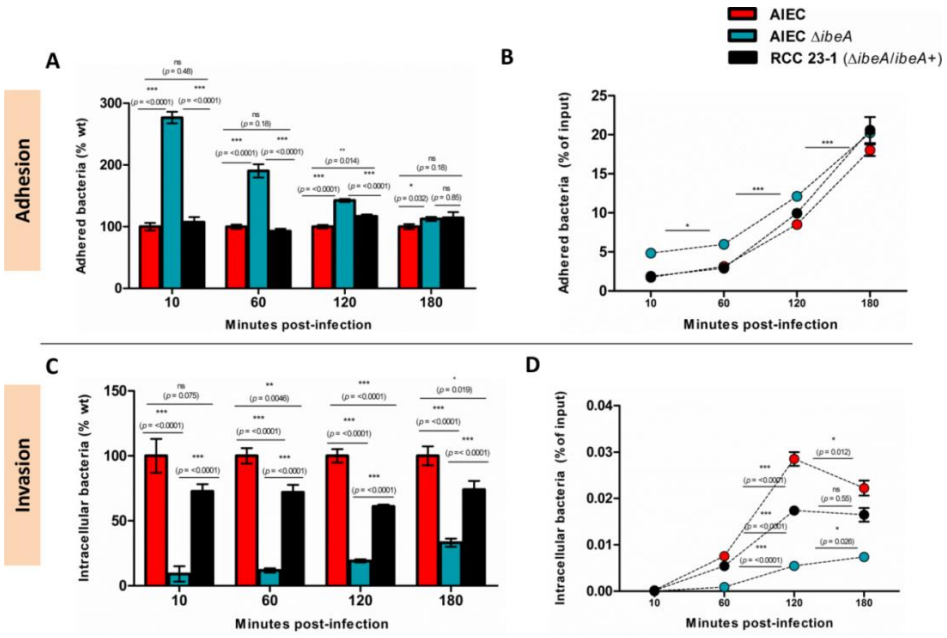


Figure 6. Kinetics adhesion and invasion of Caco-2 cells by AIEC. AIEC adhesion **(A, B)** and invasion **(C, D)** were measured in non-polarized Caco-2 cells at an MOI of 100, and bacteria was recovered and quantified at 10, 60, 120 and 180 minutes post infection. Adhesion and invasion are represented as a percentage of the initial inoculum **(B and D)**. The wt AIEC strain NRG857c is displayed in red, NRG857cΔ*ibeA* (AIECΔ*ibeA*) is in blue, while the complemented RCC 23-1 strain (Δ*ibeA*/*ibeA*+) is in black. Data are expressed as the means ± s.e. from two independent experiments (n = 10). Not significant (ns) if $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$ for comparisons between groups (One-way ANOVA followed by Tukey's multiple comparisons was used).

It is possible that in the absence of IbeA, certain adhesins are transiently over expressed, as described in the case of the fimbria Lpf. In *E. coli* O157:H7 a double *lpf*

mutant (*lpfA1* and *lpfA2*) outcompeted the wild-type in its adherence to Caco-2 cells. Contrary to what was expected, the disruption of an important factor that mediates *E. coli* adherence to the host cell did not result in a decrease in adhesion. The authors reported that, in this case, the bacteria compensated for the lack Lpf by over expressing another adhesin, curli, and this over expression was transient [107]. Further experimentation would be required to determine the up or down-regulation of additional AIEC adhesins in response to the presence or absence of IbeA. It is important to mention that even though at early time points (10 and 60 min), we observed increased adherence of NRG857cΔ*ibeA* compared to the wild-type, this was not reflected in increased invasion. Impaired invasion was constant throughout the time course evaluated.

Furthermore, it was consistently found that invasion of IEC was reduced compared to the wild-type strain NRG857c; regardless of the time point tested **(Figure 6C)**. We found that differences in invasion levels between the wild-type and the *ibeA* mutant were larger at early time points; however these differences were reduced by 3 h post infection. This suggests that the absence of IbeA delays the invasion process, since fewer bacteria will enter the IEC through time. It is possibly that the differences between wild-type and the *ibeA* mutant shortened at 3 h, because wt AIEC will reach saturation levels of invasion faster than the mutant **(Figure 6D)**.

Chapter 3.5: Role of MOI on invasion and adhesion of Caco-2 cells by AIEC

The interaction of AIEC strains with non-polarized Caco-2 cells was also studied at different multiplicities of infection (MOI). It has been reported that infections at a high MOI might result in extensive invasion, and therefore cell lysis exposing the bacteria to the extracellular gentamycin, in the case of *Listeria monocytogenes* [108]. Furthermore, it is likely that an MOI of a 100 does not represent a likely scenario in the gastrointestinal

(GI) tract for these enteric pathogens, even though this MOI is widely accepted as an experimental condition. We therefore tested different MOI, and it was found that in the strain NRG857cΔ*ibeA* adhesion seemed to be slightly increased when compared to that in the wild-type at an MOI of 1 (an increase of 24%, $p = 0.052$). This difference was not detected at an MOI of 100 **(Figure 7A)**. In the case of invasion, NRG857cΔ*ibeA* showed a decrease compared to the wild-type at all MOIs tested **(Figure 7B)**. It can be concluded that differences of invasion observed between the wild-type strain NRG857c and the *ibeA* mutant are not affected by the amount of bacteria used for infection.

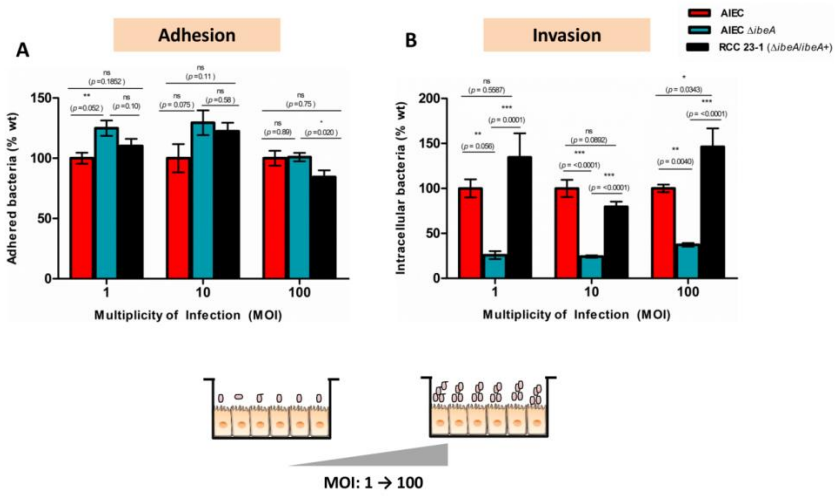


Figure 7. Role of MOI on invasion and adhesion of Caco-2 cells by AIEC. AIEC adhesion (**A**) and invasion (**B**) were measured in non-polarized Caco-2 cells at different multiplicities of infection (MOI). MOI of 1, 10 and 100 were used and bacteria recovered and quantified at 3 h post infection. The wt AIEC strain NRG857c is displayed in red bars, NRG857cΔ*ibeA* (AIECΔ*ibeA*) is in blue bars, while the complemented RCC 23-1 strain (Δ*ibeA*/*ibeA*+) is in black bars. For the invasion assays, gentamicin was added and incubation proceeded for an extra hour. Data are expressed as the means ± s.e. from two independent experiments (n = 10). Not significant (ns) if $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$ for comparisons between groups (One-way ANOVA followed by Tukey's multiple comparisons was used).

Chapter 3.6: IbeA from AIEC does not confer invasiveness to ORN172

In order to evaluate if invasiveness could be conferred to other *E. coli* strains, I transformed *ibeA* alone or combinations of *ibeRA* and *ibeRAT* (the full operon) into the strain ORN172. The strain ORN172 was selected because it has been classified as a laboratory *E. coli* strain devoid of all known adherence mechanisms, including type 1 pili and it is a non-invasive strain [87, 109]. By selecting this strain, the idea was to define whether IbeA from the strain NRG857c can render an invasive phenotype. Our results, however, showed no differences in the intracellular bacterial levels between the transformed strains and wild-type ORN172. A slight reduction, with no statistical significance is observed for all the groups (mean with SE of *ibeRA* = 80.45 ± 6.4, *ibeA* 77.12 ± 13 and *ibeRAT* 72.96 ± 2.4) when compared to the wild-type ORN172 (100%) **(Figure 8A)**. It is important to mention that the levels of intracellular bacteria (expressed as a percentage of the infection inoculum) are significantly different between the wt ORN172 and wt NRG857c (AIEC) **(Figure 8B)**. In this experiment, the culture plates were briefly centrifuged in order to allow all the bacteria to interact with monolayer, since adhesion is impaired in this strain.

The results suggested to us that the *ibeA* or the operon *ibeRAT* is insufficient to confer invasiveness to the strain ORN172. Two potential explanations for this phenomenon can be proposed. First, the bacterial genomic background of AIEC is important for the presence of the invasive phenotype, something that ORN172 does not provide. Second, that for succesful generation of an invasive phenotype the whole GimA locus might be necessary. The second alternative is less likely, since it has been reported that when non-pathogenic *E. coli* K12 HB101 was transformed with the GimA locus from NMEC (18 kb) [60], no invasive phenotype was observed. It is likely then that the

genetic makeup of AIEC and other ExPEC seem to provide the right environment to make IbeA an active player in the invasion process of host cells.

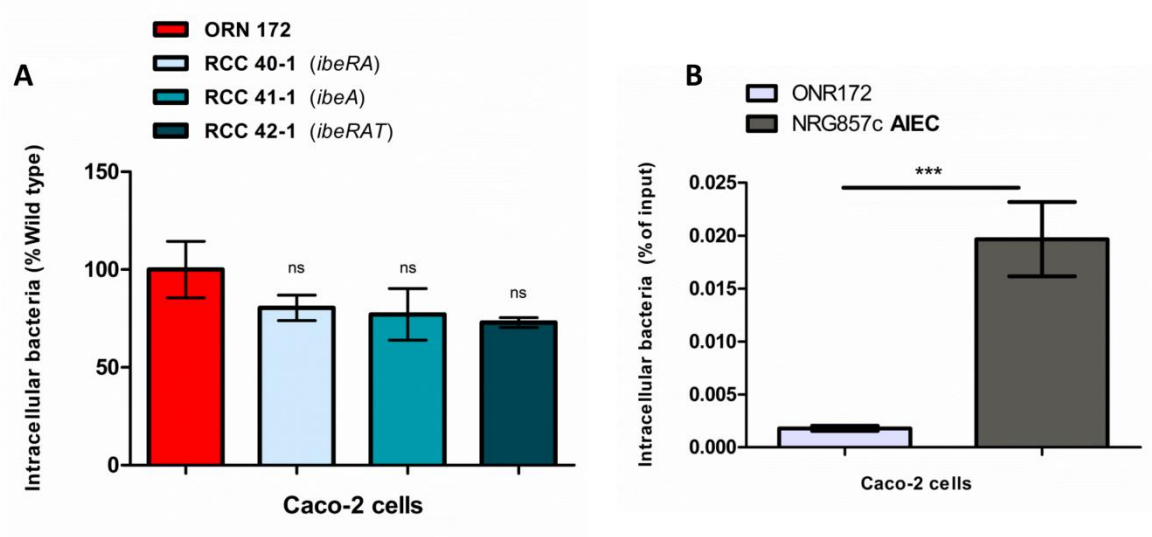


Figure 8. Complementation of ORN172 with *ibeRAT*. (A) Invasion of the strain ORN172 was measured in non-polarized Caco-2 cells at an MOI of 100. ORN172 was transformed with plasmids containing *ibeRA* (40-1) *ibeA* (41-1) and *ibeRAT* (42-1) and comparisons made at 3 h post infection. The wt strain ORN172 is displayed in red bars. **(B)** Invasion of non-polarized Caco-2 cells by ORN172 and AIEC NRG857c is expressed as percentage of the initial inoculum (input). For the invasion assays, gentamicin was added and incubation proceeded for an extra hour. Data are expressed as the means ± s.e. from two independent experiments (n = 8). Not significant (ns) if $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$ for comparisons between groups (One-way ANOVA followed by Tukey's multiple comparisons was used).

Chapter 3.7: Role of IbeA in adhesion and invasion of M cells

Another important component of the gastrointestinal epithelium is microfold cells (M cells). As mentioned before, several enteric bacteria can take advantage of M cells as portals of entry from the gut lumen to the underlying gut-associated lymphoid tissue. One of the extensively studied examples is the case of *S. enterica* serovar Typhimurium, which has been reported to not only target M cells for transcytosis [110, 111] but also to promote the transformation of primary epithelial cells to M cells, via the T3SS SopB

effector in order to enhance transcytosis across the intestinal mucosa [112]. Increased transcytosis across M cells is not the only outcome of bacteria-M cell interaction; for example *E. coli* from the serogroup O157:H7 can avoid transcytosis across M cells via the T3SS EspF effector [113, 114]. This is not closely replicable in other serogroups such as O26, indicating wide variability in the interaction of enteric bacteria with M cells [115].

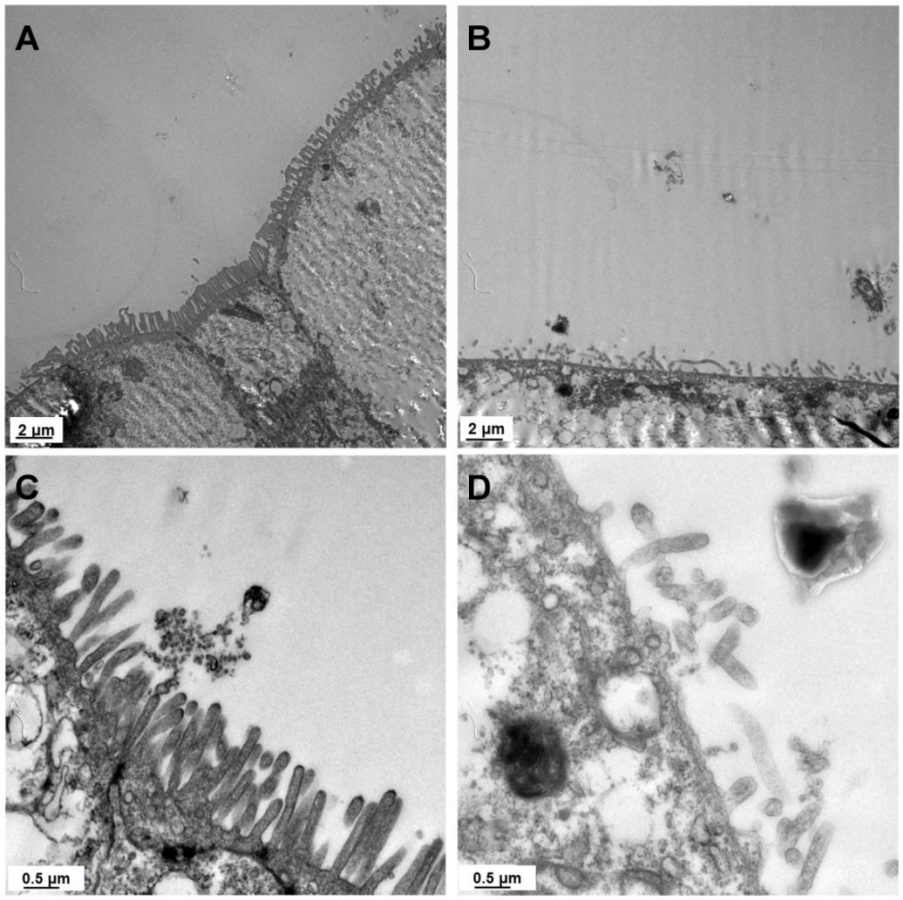


Figure 9. Generation of M-like cells. Fourteen days after polarization, Caco-2 cells were co-cultured with Raji B cells to generate M cells. Representative TEM of Caco-2 monolayers **(A)** and M cells **(B)** are shown with magnified images of selected areas underneath **(C and D).** Regions devoid of microvilli are observed in M cells. These regions were not observed in Caco-2 mono-cultures.

Few studies have evaluated the interaction of AIEC with M cells, and to date, current reports have indicated that AIEC might interact with M cells via Lpf [50]. Other virulence factors might also play a role in the interaction of AIEC with M cells, especially those involved with intracellular access, such as IbeA. Furthermore, vimentin an important M cell marker [115] is reported to be used by NMEC IbeA as a receptor and could also be used for AIEC IbeA [63, 67].

In order to evaluate the hypothesis of whether IbeA contributes to the invasion of M cells and also the transcytosis process of AIEC, an *in vitro* M cell culture was used. It is important that the primary route of crossing the M cell monolayer is believed to be transcytosis; however, it is difficult to rule out paracellular transport in this model [115]. Initially the development of M cells was confirmed by TEM as described in the methods section **(Figure 9)**. The *in vitro* M cell culture had a reduction in the surface microvilli and also increased transcytotic capability compared to results of monoculture of the progenitor Caco-2 cells.

Subsequently, bacterial adhesion and invasion were measured in M cells, which in the GI tract represent about 10% of the follicle-associated epithelium (FAE) [45]. A slight reduction was found in the adhesion levels of NRG857cΔ*ibeA* to 78.5 ± 5.2% of the wt levels (p = 0.73) **(Figure 10A)**. Additionally, the levels of intracellular bacteria recovered in the *ibeA* mutant accounted for 19.6 ± 1.05% of the wt levels (p = 0.013), while the complemented strain partially restored the phenotype to 44.4 ± 6.8% (p = 0.060) **(Figure 10B)**. Interestingly, the reduction of AIEC invasion levels due to disruption of *ibeA* in M cells (19.6 % of wt) was more prominent than the reduction observed in polarized Caco-2 cells (33.42% of wt).

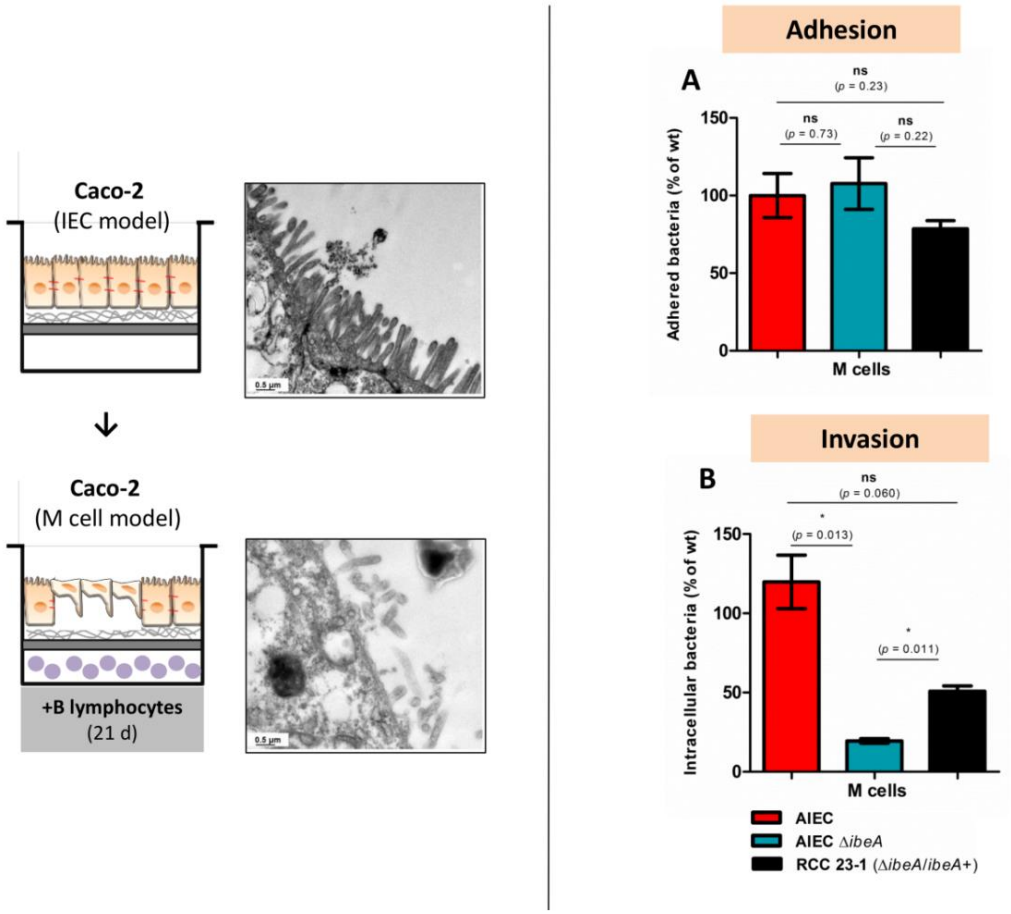


Figure 10. Role of IbeA in adhesion and invasion of M cells. AIEC adhesion (**A**) and invasion (**B**) were measured in M cells. The wt AIEC strain NRG857c is represented in red bars, NRG857cΔ*ibeA* (AIEC Δ*ibeA*) is in blue bars, while the complemented RCC 23-1 strain (Δ*ibeA*/*ibeA*+) is in black bars. For all the experiments, an MOI of 100 was used, and bacteria were recovered and quantified at 3 h post infection. For the invasion assays, monolayers were incubated with gentamicin for an extra hour. Data are expressed as the means ± s.e. from three independent experiments (n = 9). Not significant (ns) if $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$ for comparisons between groups (One-way ANOVA followed by Tukey's multiple comparisons were used). In the left side of the panel is a schematic representation of the generation of M cells. Caco-2 cells are polarized for 21 days and subsequently Raji B cells added to the basolateral chamber for 6 days to generate M cells.

Chapter 3.8: Role of IbeA in transcytosis through M cells

Transcytosis has been shown to be strain specific. For example, *E. coli* O157:H7 strain TUV 93-0 had translocation levels 4-fold higher than enteropathogenic *E. coli* O127:H6 strain E2348/69 and O26 strain ZAP1139 [115]. Scarce data are available regarding AIEC but one study showed that the transcytosis of the AIEC strain LF82 was 5-fold higher than that of a non-pathogenic *E. coli* K-12 strain [95]. Therefore, the ability of AIEC to go across M cells was analyzed next, and it was determined whether invasion of M cells by NRG857cΔ*ibeA* correlated with impaired transcytotic capability. As a control, the transcytosis was also measured across monolayers of polarized Caco-2 cells **(Figure 11A)**. At 0 h post infection, no transcytosed bacteria were found in the basolateral compartment of polarized Caco-2 monolayers or M-like cell monolayers, possibly indicating that the integrity of the monolayer is intact. When the transcytosed bacteria were measured at 1 and 2 h post infection, no significant differences were found across the groups. At 3 h post infection, the differences increased and the percentage of transcytosed bacteria was 0.65 ± 0.18% for NRG857c and 0.33 ± 0.07% for NRG857cΔ*ibeA*, ($p < 0.05$) **(Figure 11B)**. Lower levels of transcytosed bacteria in NRG857cΔ*ibeA* were expected, since the numbers of intracellular bacteria were reduced; however the reduction in transcytosis was not as marked as the reduction observed in invasion of M cells by AIEC. A possible explanation could be that since we did not observe full complementation in M cells with the strain RCC23-1, it is possible that this was also reflected by a lack of a restoration of the wild-type phenotype associated with AIEC transcytosis. Another possibility is that increased IbeA production in this strain might interfere with the process of transcytosis, causing the bacteria to stay intracellular by interacting with intermediate filaments present in M cells [63, 67, 116]. Further exploration of the mechanism of transcytosis is required, since IbeA has been reported to contribute to pathogenic bacteria crossing other biological barriers [117].

Although co-culture of Caco-2 and B cells increased the permeability of the monolayer, no significant changes in TER values between infected Caco-2 or M cell monolayers were found. Additionally, no significant TER differences were observed between groups, suggesting that differences in translocation did not correlate with TER changes, as observed when comparing NRG857c samples post infection.

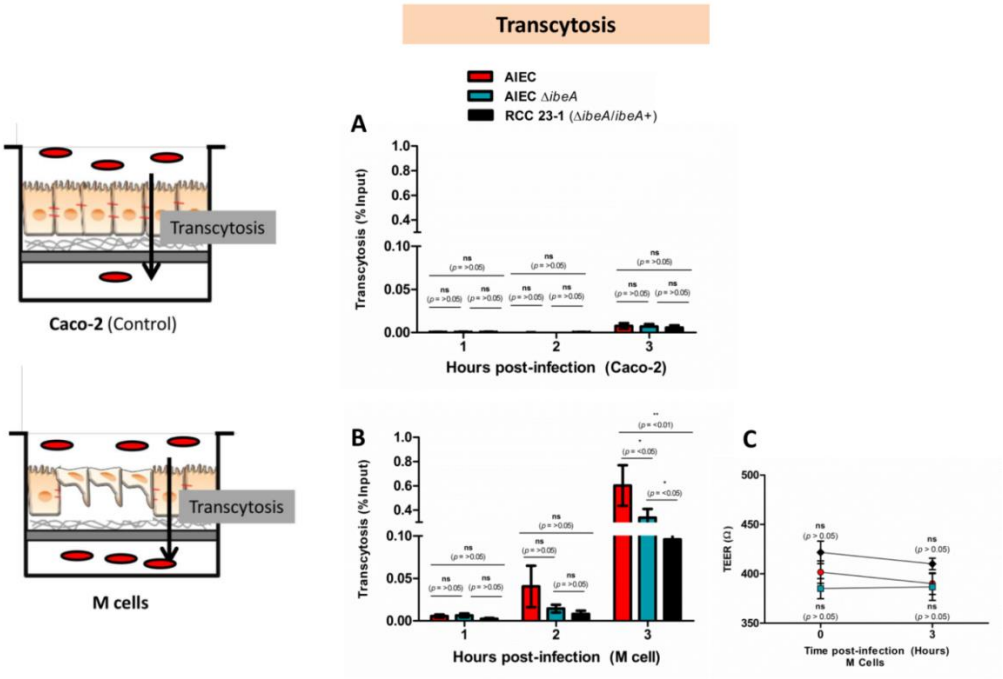


Figure 11. Role of IbeA in transcytosis through M cells. Transcytosis of NRG857 (AIEC), NRG857cΔ*ibeA* (Δ*ibeA*) and the complemented RCC 23-1 strain (Δ*ibeA*/*ibeA*+). Monolayers of 5 x 10^5 Caco-2 **(A)** or M-like **(B)** cells were infected at an MOI of 100. Bacteria were collected from the basolateral media at 1, 2 and 3 h post-infection as shown in left panel. Caco-2 monolayers were used as controls where minimal transcytosis was observed. TER was measured before infection and at the final time point (3 h) to monitor monolayer integrity. Comparisons were made at each time between groups. Numbers are expressed as a percentage of the infection inoculum (5 x 10^7 CFU). Data are expressed as the means ± s.e. from three independent experiments (n = 8). Not significant (ns) if $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$ for comparisons between groups (Two way repeated measures ANOVA followed by Bonferroni post-test was used).

The TER mean for NRG857c was 390 ± 10 Ω, for NRG857cΔ*ibeA* was 386 ± 13Ω, while for the complemented strain RCC23-1 it was 410 ± 5 Ω **(Figure 11C)**. Only a

slight reduction of around 10 Ω was observed when comparing the TER pre-infection (0 h) to post-infection (3 h) for all groups. As previously reported, all cultures in which the TER values fell below 300 Ω were excluded from experimentation [95].

Chapter 3.9: Role of IbeA in the interaction of AIEC with THP-1 macrophages

It has been shown that AIEC is taken up by macrophages where it can survive and possibly contribute to sustain inflammation [118]. To gain a more comprehensive understanding of IbeA's role in pathogenesis, survival within macrophages was determined as one of the hallmarks of AIEC infection [80, 81]. Differentiated macrophage-like THP-1 cells were used as a model [97], and bacterial uptake **(Figure 12, left panel)**, survival and replication **(Figure 12, right panel)** were measured as previously described [119]. The bacterial uptake by macrophages was determined and found to be approximately 20% of the initial inoculum (2 x 10^6 CFU) for all of the groups: NRG857c (20.69 % of input), NRG857cΔ*ibeA* (17.39%) and the control *E. coli* DH5α (22.07%) **(Figure 12, left panel)**. Survival was measured at 4, 16, 24 and 48 h post-gentamicin treatment. At 4 h post infection, the percentage of intra-macrophage survival was significantly different between the wild-type NRG857c (54%) and NRG857cΔ*ibeA* (27.31%) ($p < 0.001$). These differences were consistent throughout the time course of the experiment up to 24 h (32.42% NRG857c vs. 4.34% NRG857cΔ*ibeA*) ($p < 0.001$) and 48 h (16.525% NRG857c vs. 1.85% NRG857cΔ*ibeA*) ($p < 0.001$) **(Figure 12 right panel)**. At later time points, the survival of AIECΔ*ibeA* was similar to that of a non-pathogenic *E. coli* DH5α strain. These results are consistent with previous reports that AIEC can survive within human macrophages [80, 81]. Significant differences were found between NRG857c and NRG857cΔ*ibeA* at an early time point (4 h), as well as later (48 h), which may mean that IbeA could not only play a role in the early interaction of bacteria-macrophage, but also facilitate bacterial survival. No

intracellular replication was observed in this experiment compared to the results in previous reports [81].

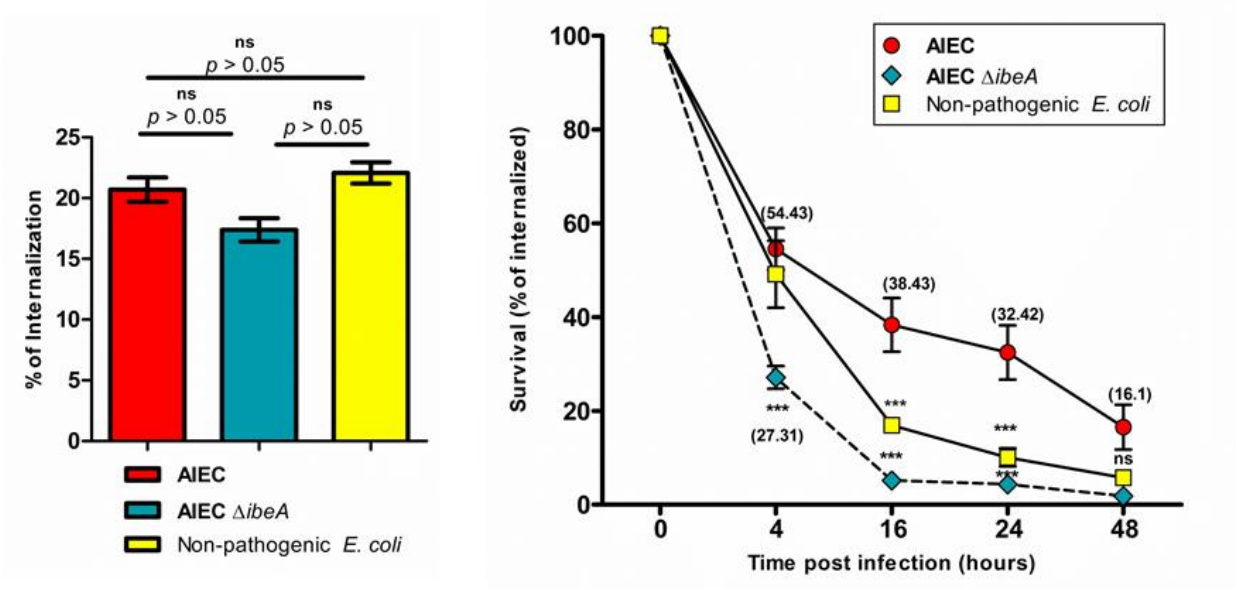


Figure 12. AIEC intra macrophage survival. THP1 macrophages were infected with AIEC, AIECΔ*ibeA* and a non-pathogenic *E. coli* DH5α at an MOI of 10 for 1 h. After infection, cells were incubated with gentamicin, and after 1h treatment, the bacterial uptake was quantified **(left panel)**. Uptaken bacteria (calculated CFU) defined after 1 h of treatment with gentamicin was at 100%. Intra-macrophage survival of bacteria was calculated at 4, 16, 24 and 48 h **(right panel)**. Data are expressed as the means ± s.e. from two independent experiments (n = 7). Two-way analysis of variance with Bonferroni post-test analysis was performed. Not significant (ns) if $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, and all of the groups were compared to wt AIEC at the different time points.

It is worth noting that further study is needed of the mechanism by which IbeA contributes to AIEC intra-macrophage survival, since a report in an APEC strain BEN2908 suggested that *ibeA* deletion makes it more sensitive to killing by H_2O_2 and implicating IbeA in the resistance to ROS response [120].

Chapter 3.10: Distribution of IbeA in other *E. coli* pathotypes

After defining that IbeA plays a role in the interaction of AIEC with the host, and determining that there is a high degree of conservation between IbeA from the AIEC strains NRG857c and LF82 as well as in the NMEC strain RS218 [69]. We decided to screen for the *ibeRAT* operon among a collection of isolates present in our laboratory database to determine if in the future, the results associated with the role of IbeA in AIEC invasion of IECs, can be reproducible with other *E. coli* pathotypes **(Figure 13) (Table 3).** Although there are limitations in screening a small sample size of pathogenic *E. coli* isolates, our results provide a starting point for further screening for *ibeRAT* in certain pathotypes such as atypical EPEC.

Pathotype	Characteristics	*ibe* RAT (+)	*ibe* RAT (-)	Total (n)
APEC		1 (33%)	2 (77%)	3
EHEC	O157:H7		2 (100%)	2
EPEC	O127:H6		1 (100%)	1
EAEC/STEC	O104:H4		3 (100%)	3
ETEC			3 (100%)	3
Shigella flexneri			1 (100%)	1
atypical EPEC		3 (37%)	5 (63%)	8
IBD isolates			4 (100%)	4
HS	Neg. Control		1 (100%)	1
# Isolates		4	22	26

Figure 13. *ibeRAT* distribution among a pathogenic *E. coli* collection. A total of 25 different strains were tested for the operon *ibeRAT*. The strains were selected as representative of different pathotypes available in our laboratory database.

In our laboratory database, we have 4 isolates from IBD patients (6604, 6380A, 6655A and 6655B) that were provided by Dr. Alexander Swidsinski from Charite Hospital, Germany [52]. It was found that these strains were all *ibeRAT* negative. This is

not surprising since a previous study in our laboratory reported that at least two of them (6604 and 6655A) were less invasive that the AIEC strain NRG857c [52]. This does not disprove the hypothesis of the association of AIEC with CD, since, as mentioned before, not all *E. coli* obtained from CD patients are classified as AIEC, and the isolates tested were just classified as IBD-associated *E. coli*. The presence of the operon was also evaluated in a collection of EAEC/STEC O104:H4 strains that caused the recent hemolytic-uremic syndrome (HUS) outbreak in Germany in 2011 [38]. It was found that these enteroaggregative pathogens that produce Shiga toxin (Stx) were *ibeRAT* negative.

Table 3. *ibeRAT* presence in a pathogenic *E. coli* collection

	Strain	**Pathotype**
1	E2348/69	EPEC
2	EDL 933	EHEC
3	86-24	EHEC
4	HS (Healthy subject)	*E. coli*
5	ETEC B7-A	ETEC
6	ETEC 214-4	ETEC
7	ETEC H10407	ETEC
8	M90T	*Shigella flexneri*
9	APEC A, Brazil	APEC
10	APEC B, Brazil (+)	APEC
11	APEC C, Brazil	APEC
12	O119N (EN10) (+)	aEPEC
13	aEPEC BA320	aEPEC
14	aEPEC BA4013	aEPEC
15	aEPEC EC292/83 (+)	aEPEC

16	aEPEC 9100-83 (+)	aEPEC
17	aEPEC 558	aEPEC
18	aEPEC 956	aEPEC
19	aEPEC 2103	aEPEC
20	2009 EL 2050 O104:H4	EAEC/STEC
21	2009 EL 2071 O104:H4	EAEC/STEC
22	2011 C3493	EAEC/STEC
23	6603B	IBD isolate
24	6380A	IBD isolate
25	6604	IBD isolate
26	6655A	IBD isolate

Of great interest was the finding that 3 strains (37%) of atypical EPEC were positive for IbeA. Atypical EPEC present the locus of enterocyte effacement (LEE) which mediates the formation of attaching/effacing lesions (A/E) [1], their central mechanism of their pathogenesis, but differ from typical EPEC in that they have lost the virulence plasmid (EAF plasmid) which codes for the Bundle-forming pili (Bfp) required for localized adherence to cultured epithelial cells [1, 3, 121]. Bfp, while not the main adherent component of atypical EPEC, does play a role in their pathogenesis, so it is possible that the strains that do not carry the EAF plasmid have developed alternative ways to interact with the host cells. Furthermore several studies have suggested that atypical EPEC are more commonly found among persistent cases of diarrhea than typical EPEC [3] and can invade IECs different from their typical EPEC [122]. A new field for exploration is to determine whether IbeA plays a role in atypical EPEC invasion and persistence.

DISCUSSION

Current knowledge regarding AIEC points to its invasion process as a topic that requires further elucidation, since no true invasin has been associated to this pathotype [6]. Availability of the complete genome sequences of the most extensively studied AIEC strains NRG857c [11] and LF82 [10] allowed the identification of a large number of putative virulence factors involved in the pathogenicity of AIEC, and the invasin from ExPEC, IbeA was among these. The results of these studies strongly support the hypothesis that IbeA plays an important role in the interaction of AIEC with the gastrointestinal epithelium, and not only IEC but also M cells, which we have confirmed with our results [84].

Our data showed a reduction in the numbers of intracellular bacteria recovered in the AIEC Δ*ibeA* strain independent of the cell model used. Nevertheless, intracellular bacteria were still found in IECs possibly meaning that other players can contribute to this process, as previously reported [37, 56]. It is also important that the invasion process was reduced in the *ibeA* mutant (fewer intracellular bacteria found throughout a time course of 3 h in the *ibeA* mutant when compared to those in the wild-type). This could be explained by either fewer bacteria entering the epithelial cells through the time course of the experiment or impaired intracellular survival within the epithelial cells; however, impaired intracellular survival is less likely, since the larger differences in invasion between the wild-type and mutant were detected during the early time points of infection (as early as 10 minutes post infection). The impairment of invasion of IEC as early as 1 h post infection by AIEC in the absence of IbeA coincided with a temporary increase in adhesion. From the results, it is difficult to conclude that this is only associated with less adhered bacteria being able to access the intracellular IEC compartment. Other studies have shown that, in the absence of an adhesin or an invasin, up-regulation of another

bacterial virulence factor can occur to compensate for this deficient interaction with the host. Interestingly, this has been reported in EHEC when the Lpf fimbriae are absent and the curli (an afimbrial adhesin) is temporarily up-regulated [107]. Even within AIEC this situation has been reported, since the deletion of the FliC subunit from flagella affects the expression of type 1 pili [37]. However, not much information is available regarding IbeA. Recently, the absence of the regulator IbeR in APEC was associated with increased expression of *ibeA*, apparently to compensate for the deleterious effect on invasion. (IbeR, however, has not been reported to be a direct regulator of IbeA) [72, 75]. It is important to study more in depth whether other virulence determinants are affected in AIEC in the absence of IbeA and to define the interplay with other members of the *ibeRAT* operon.

To further strengthen the hypothesis that IbeA plays a role in invasion of IEC by AIEC, we found intracellular bacterial levels as early as 10 minutes post infection, an indication that AIEC is an active invader, something reported before for a subset of STEC strains, which could be observed in intracellular compartments as early as 15 minutes post infection [123, 124]. IbeA seems to play a role in this active invasion process, because in the absence of IbeA, there was a drastic reduction of around 80% of the invasion levels of IEC early on during infection. Another finding associated with AIEC invasion was that the number of internalized bacteria recovered in the wild-type strain peaked around 3 h post-infection. Other enteric pathogens such as *S.* Typhimurium, can reach IEC intracellular levels higher than AIEC only after 1 h post infection (up 6% of the initial inoculum) [125], which culminates in the destruction of the cell monolayer [126]. This could be detrimental for a pathogen reported to be involved in chronic pathology such as CD [5, 14, 21]. Whether the plateau observed in the number of intracellular AIEC at 3 h post infection implies that further damage of the monolayer is prevented, is a hypothesis that could be explored in the future.

The findings in my study also are innovative because IbeA allows AIEC to colonize IEC (intestinal epithelial cells). IbeA has been previously reported to contribute to the invasion of BMEC (brain microvascular endothelial cells), and chicken embryo fibroblasts. In these cells, it was reported that IbeA mediates *E. coli* intracellular access via interaction with Vimentin and PSF [63, 64, 67]. Caco-2 cells could present a very different scenario since more than one group has reported that they do not express vimentin [63, 116]; however, AIEC invasion was still detected. It is possible then, that AIEC invasion of Caco-2 is solely dependent of PSF (the other identified receptor), or another receptor needs to be identified. Another report [127] suggested that two binding bands were present in Caco-2 lysates (IBP200 with a MW of ≈ 200 kDa and IBP90 with a MW of ≈ 90 kDa). Further research into this topic has not had any follow up by the same group. The results obtained in this study with the AIEC strain NRG857c regarding the invasion of Caco-2 cells via IbeA currently support the idea that a receptor other than the known IbeA receptors might be used by AIEC to access intestinal epithelial cells. It is possible that vimentin is relevant in the interaction with M cells, which express this protein [112].

While the foregoing discussion relates wholly to my primary hypothesis, based on the conclusions obtained in this study, it is worth mentioning that epithelial cells under pathological conditions could express vimentin (which is not normally expressed), such as in the case of tubular epithelial cells in the kidneys [128], and more recently reported in pathologies such as CD [129]. Vimentin, as a potential receptor for AIEC invasion, has only been studied in the strain CUICD541-10, although the model used (embryonic kidney cells) differed from all of the previous characterizations of AIEC invasion, mainly those studied in IECs [6, 37, 56]. It would be interesting to study whether, upon AIEC invasion, IECs can express vimentin as reported in the pathologies mentioned above. If Caco-2 vimentin expression in response to AIEC is proved, an alternative hypothesis

could be proposed indicating that under pathologic conditions, IECs will express vimentin by temporarily facilitating AIEC invasion through IbeA, in a similar fashion to that described in the interaction of CEACAM6 with AIEC type 1 pili [26, 27].

Additionally, the contribution of IbeA to the invasion process of AIEC is likely to be different than in ExPEC strains such as NMEC and APEC, because it is believed that a coordinated action with other invasive determinants such as CNF1 (cytotoxic necrotizing factor 1) and IbeB occur [64] [130]. While IbeB has been reported to be widely distributed among pathogenic and non-pathogenic *E. coli* (it was reported present in *E. coli* BL21 and MG1655) [131], CNF1 distribution is limited. In NMEC it has been reported that CNF1, IbeA and OmpA act in concert to activate a subset of Rho GTPases (RhoA, Rac1 and Cdc42), inducing changes in the host that mediate the invasion of brain endothelial cells (BMEC) and subsequent penetration of the blood-brain barrier. While CNF1 was capable to activate the three Rho GTPases mentioned, IbeA was just responsible for the activation of Rac1 [132]. Therefore, the exact mechanism by which IbeA contributes to AIEC invasion of IECs requires further clarification.

Regarding the association of IbeA with the transcytosis process through M cells, the results of this study have also shown that IbeA plays an important role. Several pathogens have been shown to take advantage of the host to fulfill this purpose, such as *Y. enterocolitica* [133] which selectively invades M cells, or *Salmonella* [110, 112], which stimulates the conversion of intestinal epithelial cells into M cells to facilitate its transcytosis. On the other hand, *E. coli* O157:H7 impairs transcytosis to favor colonization [113-115], primarily through T3SS effectors. AIEC, on the other hand, does not contain a T3SS apparatus [10, 11], and the fimbrial adhesin Lpf has been reported to play a role in the interaction of AIEC with M cells [50, 51]. The finding that in the absence of IbeA, transcytosis through M cells was reduced, has led others to suggest that

AIEC might have more than one mechanism to interact with M cells [50, 51]. It is possible then, that one mechanism of AIEC transcytosis through M cells is a receptor-mediated process via IbeA, instead of a passive process such as endocytosis or macropinocytosis. Identification of a receptor in M cells requires further study; however, vimentin seems to be the prime candidate [116]. This process might comprise an alternative route for AIEC access to the underlying immune cells in the GI tract, besides invasion of IECs [6, 14] . Subversion of a biological barrier is a key event in the case of AIEC for the initiation and perpetuation of inflammation [21], and these studies show that AIEC might have more than one mechanism to achieve this goal [50, 56].

It is important to emphasize that not all biological barriers present the same challenge for an invasive pathogen because of intrinsic differences, such as those found in the placenta, blood-brain barrier and the GI tract [117]. While BMEC shares similarities with the GI barrier, such as the presence of tight junctions, surface molecules are expressed differentially in both anatomical barriers [63, 116, 129], and they can exert a functional difference if they are used as bacterial receptors [134]. Therefore, the invasin IbeA is presented with a unique and different cellular scenario to exert its invasive properties.

In this study we have also addressed the other cell component targeted by AIEC upon invasion of the intestinal epithelium, the underlying macrophages. The majority of AIEC studies have been carried on macrophages of murine origin [80], while in this study we have addressed the interaction with human-derived macrophages (THP-1). Differently from what was found with strain LF82, in our AIEC strain NRG857c, we did not find the levels of intracellular survival reported previously [80], which could be a reflection of the different cell model. What we did found in this study is the ability of IbeA to contribute to the intracellular survival of AIEC within macrophages. In its

absence, we observed that AIEC behaved more similarly to a non-pathogenic laboratory *E. coli* DH5α strain.

We have also replicated previous results indicating that the bacterial genetic background in which a virulence factor is present does have an important role in the overall phenotype. For example, our experimental evidence showed that transforming the strain ORN172, which is considered a non-invasive organism and found at significantly lower numbers than AIEC NRG857c, was not rendered invasive in presence of the *ibeRAT* operon. Similar findings have been reported regarding IbeA in other pathotypes. Cloning the whole GimA locus from an NMEC strain in non-pathogenic *E. coli* did not increase invasion [68]. It could be proposed that within each *E. coli* pathotype, there is a complex interplay leading to the functional expression of this invasin. It is worth mentioning that while AIEC is in the same phylogenetic group than ExPEC, they do have several differences at the genetic and virulence levels [10, 11]), meaning that the contribution of IbeA to the pathogenesis of AIEC could be different from that previously described. Furthermore, in this study we have detected the presence of the operon *ibeRAT* in a subset of atypical EPEC, which is associated with more persistent cases of diarrhea than the typical EPEC counterpart [3]. It is possible that atypical EPEC might have acquired other virulence determinants associated with their invasive virulent traits.

Although it is true that other virulence factors are also important for the interaction of AIEC with the host, such as Lpf [50], type 1 pili [26, 37] and outer membrane proteins (OMPs), here we define IbeA as an important invasin in the AIEC pathotype and emphasize that IbeA is a true invasin, based on the extensive evidence that its contribution to adhesion is negligible and its distribution limited to the pathotypes that have been reported to be able to penetrate host barriers in order to cause disease [117]. The invasion process of AIEC has been described to be actin- and microtubule-dependent

[5]. Since bacteria can employ several strategies to manipulate the same components of the host, now that IbeA has been reported as an AIEC invasin, it would be interesting to determine the role this invasin plays in the process [135].

RESULTS AND DISCUSSION

Chapter 4: Participation of the invasin IbeA in an *in vivo* animal AIEC colonization model

INTRODUCTION

Most of the discussion in previous chapters centered on the interaction of AIEC with intestinal epithelial cells, M cells and macrophages. Upon characterizing the role of this invasin *in vitro*, the next step was to define whether IbeA played a role in an *in vivo* animal model of colonization, especially if in the absence of IbeA the reported contribution of AIEC to inflammation in the mouse GI tract is altered. Before discussing the results obtained in this section, the limitations of using animal models in the study of AIEC are briefly reviewed.

One of the most important limiting factors in the study of AIEC interaction with the host has been the lack of a suitable animal model in which long-term colonization can be evaluated. The models used for long-term colonization by AIEC have involved the administration of a colitogenic chemical, such as the detergent dextran sulfate sodium (DSS). However, it is widely accepted that DSS is toxic to epithelial cells and causes defects in the epithelial barrier integrity [136]. In models like the DSS-induced-colitis model, the damage of the epithelial barrier, prior to bacterial infection impairs the evaluation of the interaction of AIEC with the intestinal epithelium.

However, in a recently newly described mouse model, it has been suggested that AIEC might play a role as an instigator of colitis in either a normal or susceptible host. In germ-free mice with an innate immune deficiency, such as a lack of the flagellin

receptor (Toll-like receptor 5), the colonization by the AIEC strain LF82, which was detectable up to 10 days, resulted in chronic colitis, persisting for months [137]. This observation was not exclusive for mice with an altered immune response since the AIEC strain LF82 was found able to persist not only in transgenic mice expressing the human receptor CEACAM6 [26], but also in a wide array of conventional mouse strains (CD-1, DBA/2, 129e, and C3H) that were streptomycin-treated, a procedure used to reduce the animal normal intestinal flora, thereby decreasing competition for the infection from the *E. coli* strain [138]. These mouse strains that were streptomycin-treated can get both AIEC prototype strains (LF82 and NRG857c) persisting up to 28 days, and the colonization was associated with a chronic pro-inflammatory response in the ileum, cecum and colon as seen by elevated histopathological scores [98].

This later CD-1 (ICR) mouse model in which strain NRG857c was capable of colonizing and persisting in the gut (small intestine, cecum and colon) causing inflammation and pathology up to 28 days [98], we found most suitable to evaluate the effect of the *ibeA* deletion on colonization and inflammation because (a) the same strain was used to create the *ibeA* mutation and (b) the integrity of the intestinal epithelium was not compromised by a detergent prior to infection.

RESULTS

Chapter 4.1: Effect of IbeA on AIEC intestinal colonization of mice

A model of AIEC colonization *in vivo* has recently been reported, in which our AIEC strain NRG857c persisted and caused inflammation in the intestines of conventional mouse strains [98]. To determine the contribution of IbeA to AIEC-mediated colonization and inflammation *in vivo*, two different time points were established, at both the early stage of colonization (4 days post-infection) and at the late stage (14 days post-infection). The late stage time point was chosen to establish whether IbeA participates after colonization is established, and most of the initial inoculum (infectious dose) is shed. In all of the experiments, mouse groups treated with PBS or infected with *E. coli* K12 MG1655 [11] were used as negative controls.

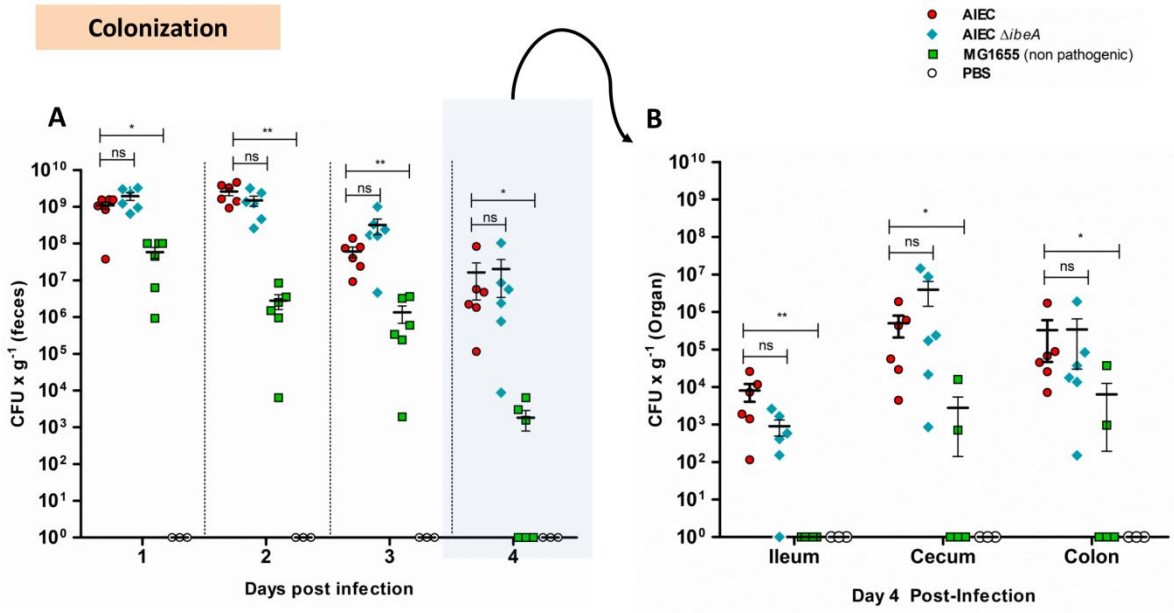


Figure 14. AIEC bacterial counts in feces and intestines during the early stage of colonization. Groups of six female, streptomycin pre-treated CD-1 (ICR) mice were infected with 1 x 10^9 of NRG857 (AIEC), NRG857c Δ*ibeA* (Δ*ibeA*), MG1655 (non pathogenic K12 *E. coli*) or PBS (negative control) via oral gavage as described in the methods section. Feces were collected daily, and sections of the ileum, cecum and colon were homogenized and CFU recovered at day 4 post infection **(A)**. Solid lines indicate the arithmetic mean for each of the infected groups. The CFU levels per organ and in the feces are also reported **(B)**. Data are expressed as the means ± s.e. from six mice per group, representative of one experiment. Not significant (ns) if $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$ compared with the wt strain NRG857c. (Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparisons tests was used.)

At four days post infection, no significant differences were found in the bacterial levels across the intestines between the wild-type strain NRG857c and mutant NRG857cΔ*ibe.* When comparing the NRG857c strain with *E. coli* MG1655, statistically significant differences were found in the ileum ($P \leq 0.01$, ANOVA p value = 0.0028), cecum ($P \leq 0.05$, ANOVA p value = 0.0059) and the colon ($P \leq 0.05$, ANOVA p value = 0.0160) **(Figure 14A)**. As expected, MG1655 was less efficient at colonizing and persisting than NRG857c. The differences observed between the bacterial levels were not attributed to changes in the percentage of infected mice. It was only in day 4 that the group infected with MG1655 had 50% of the mice with non-detectable bacteria **(Figure 14B).** The differences between MG1655 suggest that our AIEC strain NRG857c as reported previously does not behave like regular non-pathogenic *E. coli*, strengthening the fact that while present in the gastrointestinal under non-pathologic conditions, this bacteria does not behave like a commensal.

Next, the IbeA contribution to the late stage of colonization was determined. Because the bacterial numbers did not have a normal distribution, the mean number of CFU per gram of feces was compared against the wild-type AIEC strain NRG857c at each time point. There were no statistically significant differences between mice infected

with AIEC NRG857c compared to NRG857cΔ*ibeA* during the course of infection. In contrast, differences between AIEC NRG857c and MG1655 were statistically significant for the first 4 days of the study **(Figure 15A),** and statistical analysis could not be applied at later time points because mice cleared MG1655 from the intestine. For example, at day 8 post infection, bacterial levels of NRG857c were 1.56 x 10^3 CFU/gram of feces versus no detectable levels for MG1655. All of the colonization values for days 4 and 14 are listed in **Table 4.** In all cases, the bacterial levels in the feces were consistent with those found across the intestine. In each of the intestinal sections, NRG857c and NRG857cΔ*ibeA* had similar levels of colonization, while strain MG1655 was not detected in the intestine at 14 days post infection **(Figure 16).**

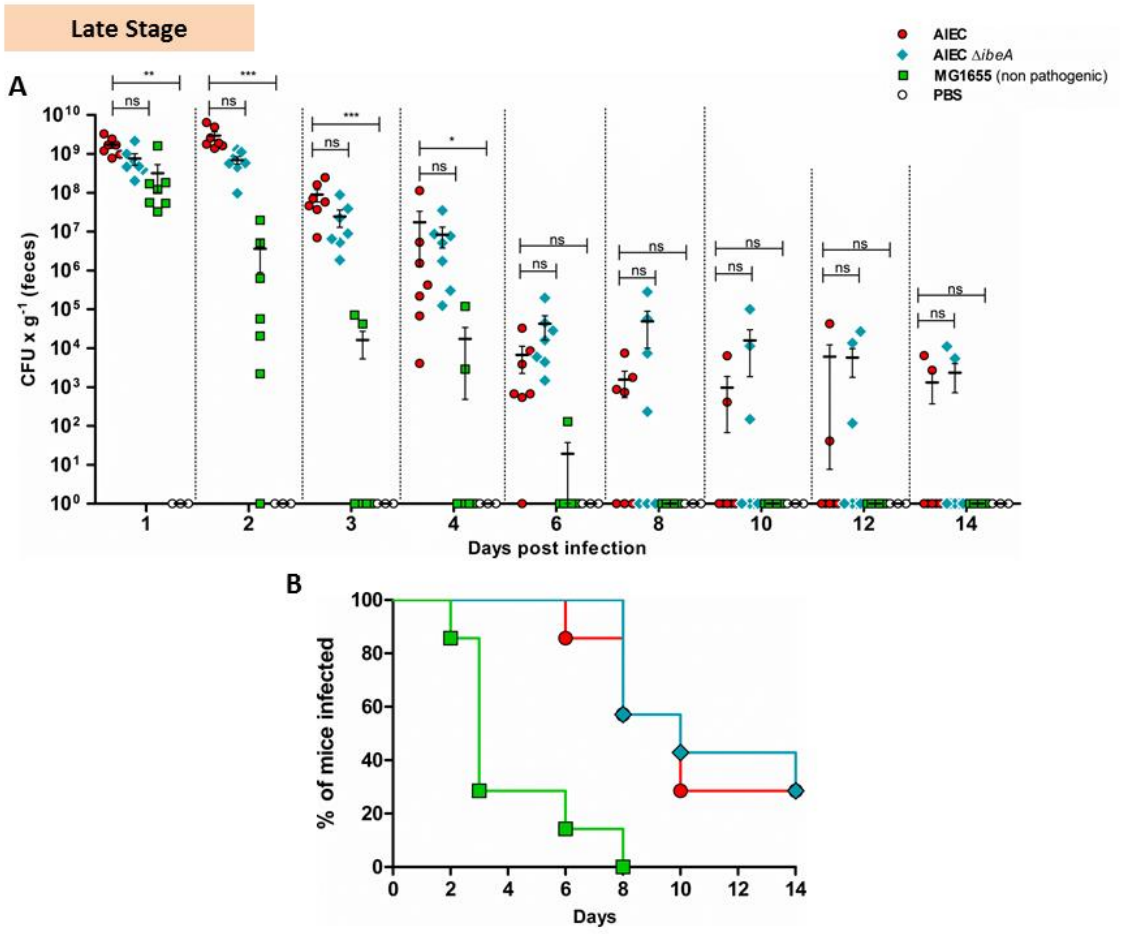


Figure 15. AIEC bacterial counts in feces during late stage of colonization. Groups of seven female, streptomycin pre-treated CD-1 (ICR) mice were infected with 1 x 10^9 of NRG857 (AIEC), NRG857c Δ*ibeA* (AIEC Δ*ibeA*), MG1655 (non-pathogenic *E. coli* K12) or PBS (negative control) via oral gavage. Feces were collected daily and subsequently every other day up to 14 days **(A)**. The percentage of mice that were infected, based on our threshold of bacteria detection, was also determined. Results are expressed as the percentage of the infected mice. The resulting curves were compared via a Log-rank test **(B)**. Data are expressed as the means ± s.e. from seven mice per group, representative of one experiment. Not significant (ns) if $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, and *** $p \leq 0.001$ compared against the wt strain NRG857c. (Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple comparisons tests was used.)

At 14 days post infection, detectable bacteria were found in the feces of 28% of mice from the group infected with NRG857c, while the group infected with NRG857cΔ*ibeA* was at 37%. Although no significant differences were found between NRG857c and NRG857cΔ*ibeA* ($p = 0.83$, Log-rank test), differences in the curves were statistically significant when compared to those from strain MG1655 ($p = 0.002$ and $p = 0.001$ respectively, Log-rank test) **(Figure 15B).** These results suggest that the absence of IbeA does not seem to affect the ability of NRG857c to persist within the mouse intestine.

Table 4. Bacterial shedding in feces and colonization levels

	CFU x g^{-1} (feces)		
	NRG857c	**NRG857c Δ*ibeA***	**MG1655 (K12 *E. coli*)**
Day 1	**1.72E+09** ± 3.31E+08	**7.61E+08** ± 2.50E+08^{ns}	**3.17E+08** ± 2.15E+08**
Day 2	**2.69E+09** ± 7.37E+08	**4.03E+08** ± 1.52E+08^{ns}	**3.64E+06** ± 2.76E+06***
Day 3	**8.97E+07** ± 3.20E+07	**2.46E+07** ± 1.17E+07^{ns}	**1.63E+04** ± 1.10E+04***
Day 4	**1.74E+07** ± 1.61E+07	**8.40E+06** ± 4.64E+06^{ns}	**1.74E+04** ± 1.69E+04*
Day 6	**6.73E+03** ± 4.52E+03	**4.24E+04** ± 2.62E+04^{ns}	**1.84E+01** ± 1.84E+01^{ns}

Day 8	**1.56E+03** ± 1.01E+03	**4.96E+04** ± 3.97E+04^{ns}	**ND**^{ns}
Day 10	**9.74E+02** ± 9.08E+02	**1.60E+04** ± 1.41E+04^{ns}	**ND**^{ns}
Day 12	**6.08E+03** ± 6.07E+03	**5.77E+03** ± 3.99E+03^{ns}	**ND**^{ns}
Day 14	**1.31E+03** ± 9.38E+02	**2.63E+03** ± 1.65E+03^{ns}	**ND**^{ns}
	CFU x g^{-1} (organ)		
	NRG857c	**NRG857c Δ*ibeA***	**MG1655 (K12 *E. coli*)**
Day 4 (Ileum)	**8.08E+03** ± 4.02E+03	**9.06E+02** ± 4.19E+02^{ns}	**ND****
Day 4 (Cecum)	**5.03E+05** ± 2.95E+05	**3.95E+06** ± 2.54E+06^{ns}	**2.79E+03** ± 2.65E+03*
Day 4 (Colon)	**3.27E+05** ± 2.81E+05	**3.42E+05** ± 3.12E+05^{ns}	**6.36E+03** ± 6.17E+03*
Day 14 (Ileum)	**1.29E+02** ± 1.05E+02	**2.44E+04** ± 2.17E+04^{ns}	**ND**^{ns}
Day 14 (Cecum)	**7.08E+02** ± 5.17E+02	**3.11E+03** ± 2.41E+03^{ns}	**ND**^{ns}
Day 14 (Colon)	**4.19E+02** ± 3.10E+02	**4.14E+02** ± 3.56E+02^{ns}	**ND**^{ns}
	Values represent the mean ± SE at each time point		
	* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ denote statistical significance when compared to NRG857c		
	ND = non detectable levels of bacteria		

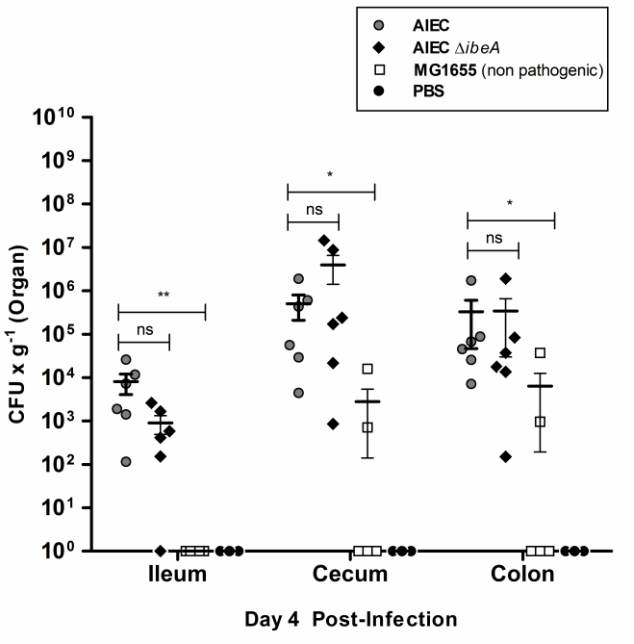


Figure 16. AIEC bacterial counts in the intestine during the late stage of colonization. Groups of seven female, streptomycin pre-treated CD-1 (ICR) mice were infected with 1 x 10^9 of NRG857 (AIEC), NRG857c Δ*ibeA* (Δ*ibeA*), MG1655 (non-pathogenic *E. coli* K12) or PBS (negative control) via oral gavage. Sections of the ileum, cecum and colon were homogenized, and CFU recovered at day 14 post infection. Data are expressed as the means ± s.e. from seven mice per group, representative of one experiment. Not significant (ns) if $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, *** and $p \leq 0.001$ compared against the wt strain NRG857c. (Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple comparisons tests was used.)

Chapter 4.2: AIEC infection and inflammatory associated markers

The local inflammatory immune response was measured in the cecum and colon of infected animals at 14 days post infection. The inflammatory mediator IFN-γ was selected because it is known to be elevated in the lamina propia of CD lesions [139]. Further, in a model of inflammation mediated by the AIEC strain NRG857c, IFN-γ was elevated in the colon and the cecum of infected CD-1 (ICR) mice [98]. Mice infected with NRG857c had significant increases in the levels of IFN-γ in the cecum **(Figure 17A),** but not in the colon. The levels of IFN-γ in the cecum of animals infected with

NRG857c were 123.4 ± 36.5 pg/ml, while those in the PBS control group were 10.7 ± 0.79 pg/ml ($p = 0.021$), which corresponds to an increase of more than 10-fold. The colon presented a very different scenario **(Figure 17B)**; with levels of IFN-γ detected in the group infected with AIEC of 46.6 ± 13.8 pg/ml and no significant amounts in the other groups.

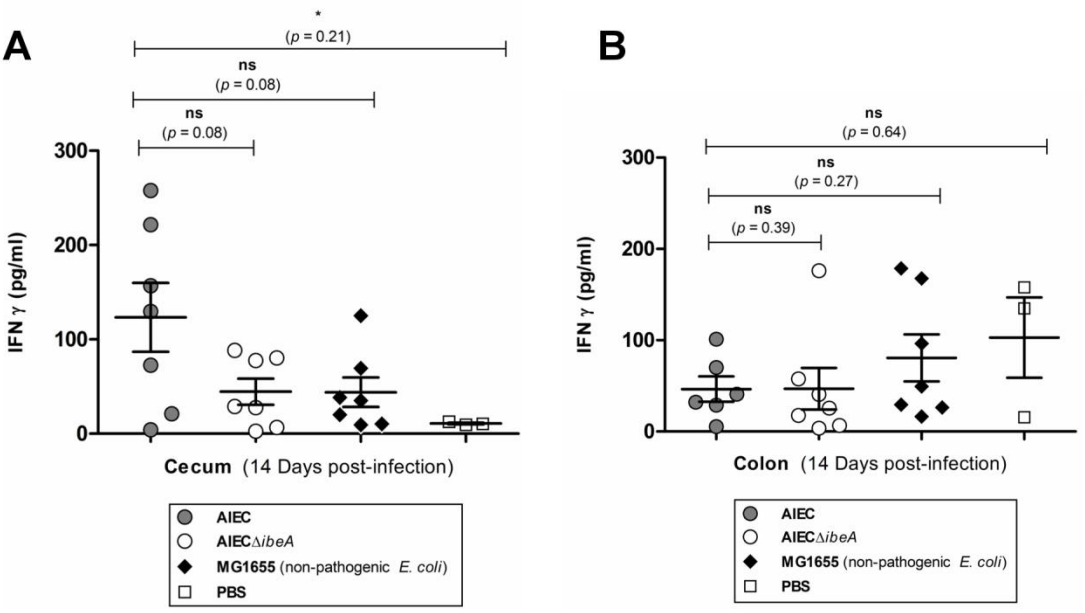


Figure 17. IFN-γ local inflammatory response in the colon and cecum following infection with AIEC. Groups of seven female, CD-1 (ICR) mice were infected with NRG857 (AIEC), NRG857c Δ*ibeA* (Δ*ibeA*), MG1655 (non pathogenic *E. coli* K12) or PBS (negative control), and tissue sections of the colon and cecum were obtained 14 days post infection. IFN-γ levels were measured by an ELISA in the cecum **(A)** and colon **(B)**. Data are expressed as the means ± s.e. from seven mice per group, representative of one experiment. Not significant (ns) if $p > 0.05$, *$p \leq 0.05$, and **$p \leq 0.01$ compared against the wild-type strain NRG857c. (Unpaired t-test with Welch's correction, due to differences in the variance between samples population, was used.)

When comparing the IFN-γ levels in the groups infected with AIEC NRG875 and NRG857cΔ*ibeA*, a slight reduction was found in the cecum (from 123.4 ± 36.5 pg/ml to 44.5 ± 13 pg/ml), but the reduction did not reach statistical significance ($p = 0.08$).

However, 5 of the 7 mice had IFN-γ values higher than 100 pg/ml in the cecum, which was not observed in any of the mice infected with NRG857cΔ*ibeA*.

Furthermore, the IFN-γ values observed in the NRG857cΔ*ibeA* (44.5 pg/ml) group resembled more those observed in the group infected with the non-pathogenic MG1655, (43.9 ± 15 pg/ml; p = 0.97) **(Figure 17A)**. The results suggest that, in the absence of IbeA, the IFN-γ response in the cecum was diminished, with NRG857cΔ*ibeA* resembling more closely the inflammatory pattern observed with the non-pathogenic MG1655. In contrast, the IFN-γ response observed in the colon between the AIEC wild-type and NRG857cΔ*ibeA* did not differ drastically in their means (46.46 ± 13 pg/ml versus 46.84 ± 22 pg/ml, p = 0.39) **(Figure 17B),** suggesting that the IFN-γ inflammatory-associated response against AIEC in the colon was not as pronounced as in the cecum. Overall, increased levels of IFN-γ were observed in the cecum when compared to those in the colon in response to AIEC NRG857c, as previously reported [98].

At day 4 post infection, the levels of TNF-α were below the limits of detection in the cecum and the colon for all of the groups (< 6 pg/ml) (data not shown). At day 14 post infection, however, TNF-α levels were detectable in the colon but not in the cecum **(Figure 18).** There was extensive dispersion of the data within groups, and although the mean value for the wt AIEC NRG857c was higher than those of the *ibeA* mutant and MG1655, none of them differed significantly from the PBS control group. Furthermore, the levels of TNF-α recovered in this study were significantly lower than those observed in a previous report [98].

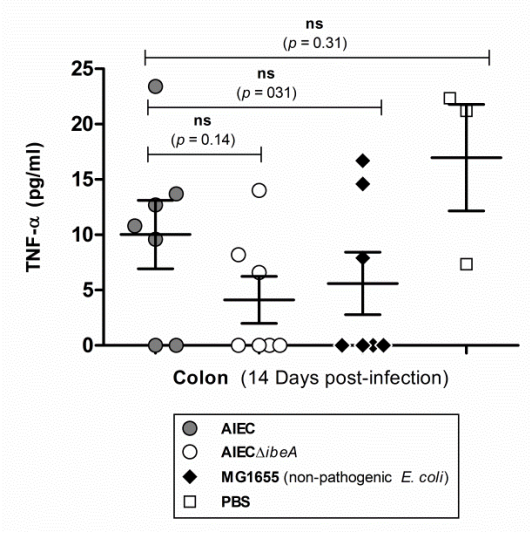


Figure 18. TNF-α levels in the colon and cecum following infection with AIEC. Groups of seven female, CD-1 (ICR) mice were infected with NRG857 (AIEC), NRG857c Δ*ibeA* (Δ*ibeA*), MG1655 (non pathogenic *E. coli* K12) or PBS (negative control), and tissue sections of the colon were obtained 14 days post infection. TNF-α levels were measured by an ELISA in the colon. Data are expressed as the means ± s.e. from seven mice per group, representative of one experiment. Not significant (ns) if $p > 0.05$, *$p \leq 0.05$, and **$p \leq 0.01$ compared against the wild-type strain NRG857c. (Unpaired t-test with Welch's correction, due to differences in the variance between samples population, was used.)

Chapter 4.3: AIEC pathology in the murine intestine

Histological analysis of hematoxylin and eosin-stained small intestine, cecum and colon was performed in all seven animals from each AIEC-infected group, even if bacteria were not detected after 14 days **(Figure 19A)**. In the ileum of animals infected with AIEC NRG857c, a high pathology score (4.5) was obtained as a result of the damage in the epithelium. Desquamation of the surface epithelium **(Figure 19A)** with focal ulceration of the mucosa and complete loss of villi and crypts were observed. The damage in the lumen of the ileum was not as marked as that observed in the surface epithelium (black bars vs. dark grey bars in **Figure 19A)**. In the group infected with NRG857cΔ*ibeA* (2.33, ileum pathology score), a disruption of the surface epithelium

with desquamation and no major damage in the lumen was observed. The PBS-treated group consisted mostly of unremarkable mucosa with a slightly hypercellular lamina propia (0.5, ileum pathology score). Similar results were observed in the cecum with the difference that the lumen showed a moderate-to-dense presence of necrotic epithelial cells only when infected with NRG857c (dark grey bars in **Figure 19B, E)**. The pathology observed in the colon, consistent with our IFN-γ results, was shown to be unremarkable, and the differences in the pathology scores means were not drastically different between the AIEC-infected groups (1.50 and 1.167) or with PBS (1.50 vs. 0.75) **(Figure 19C)**. This result contrasted with those observed in the small intestine (4.5 vs. 0.5) and colon (4.16 vs. 1.75). When comparing the pathology scores between animals infected with NRG857c and NRG857cΔ*ibeA*, we found lower numbers for the latter group in the ileum (4.5 vs. 2.33) and the cecum (4.16 vs. 1.83), but not in the colon (1.50 vs. 1.167). Overall, the results showed that in the mice colonized with NRG857cΔ*ibeA*, there is a reduction in the damage to the intestinal tissue, particularly in the ileum and cecum. This result is consistent with that previously shown with AIEC infections leading to tissue pathology [11] and strengthens the role of AIEC bacteria as a promoter of inflammatory disorders in the gastrointestinal tract [54].

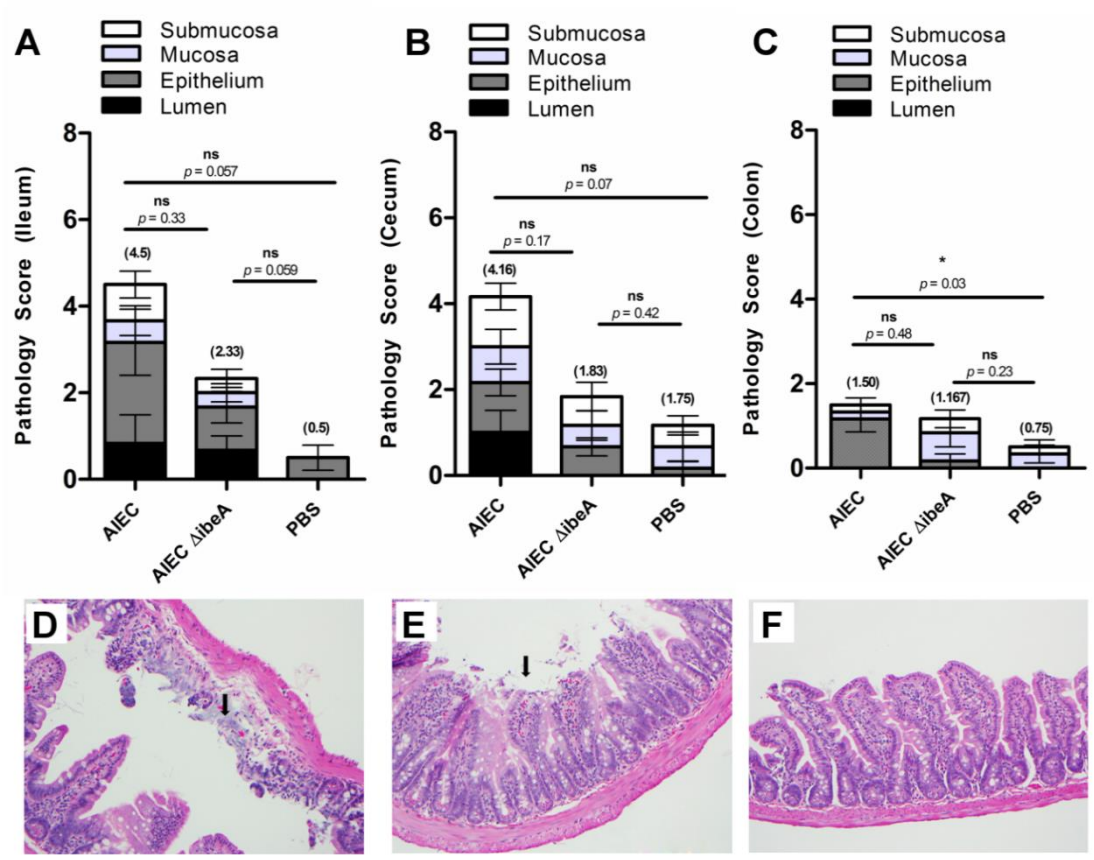


Figure 19. Ileal, cecal and colonic pathologies following AIEC infection. The pathology scores were calculated for the **(A)** small intestine, **(B)** cecum and **(C)** colon at 14 days post infection after staining with hematoxylin and eosin. ICR (CD-1) mice infected were as follows: AIEC (n = 6), AIECΔ*ibeA* (n = 6) or PBS (n = 3). Scores represent an average of three views per section, and data are expressed as the means with standard deviation for each group. Panels **(D)**, **(E)** and **(F)** are representative small intestinal sections that were stained with hematoxylin and eosin and used to calculate pathology scores. Ileum of CD-1 (ICR) infected with AIEC **(D)**, AIECΔ*ibeA* **(E)**, and PBS control **(F)** are displayed. The extensive damage to the intestinal epithelium can be observed (black arrows). Data are representative of one experiment. The pathology scores for each intestinal segment with each bacteria are presented between parenthesis in each figure. Pathology scores means were compared by using a One-way ANOVA followed by Tukey's multiple comparisons. Not significant (ns) if $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, and $*** p \leq 0.001$ for comparisons between groups were established.

DISCUSSION

In this murine model of infection, we found that AIEC was able to persist within the GI tract for up to 14 days. We found that AIEC can colonize the gut of conventional mice more efficiently than non-pathogenic *E. coli,* strengthening the hypothesis that AIEC presents characteristics that allow it to colonize the GI tract more efficiently than *E. coli* not classified as pathogenic. Further, AIEC colonization was not the direct result of genetic deficiencies of the host that facilitate AIEC persistence, such as over-expression of CEACAM6 in the gut [118, 140]. Colonization of AIEC was not located to one exclusive section of the gut, since bacteria were recovered from the small intestine, cecum and colon, indicative that AIEC has the ability to interact with different intestinal sections.

Although the absence of IbeA did not cause a significant reduction in murine intestinal colonization, an alternative hypothesis indicates that AIEC is equipped with additional adhesins and/or invasins that mediate intestinal interaction. While colonization was not affected, we found that IbeA is important for increased pathology in the cecum of mice as demonstrated by increased IFN-γ levels. The reduction in IFN-γ secretion in the absence of IbeA is possibly a consequence of impaired interaction with specific cells of the innate immune system, such as macrophages, and not as a consequence of changes in colonization of the intestinal epithelium.

In conditions such as CD, altered patterns of cytokine production by immune cells of the lamina propia have been reported. A functional relevance and association to the clinical pathology observed in CD has been attributed to these cytokine-altered patterns. For example, IFN-γ has been primarily associated with CD. This suggests that innate lymphoid cells underlying the lamina propia in the gut are an important source of IFN-γ, which is increased in patients with CD, but not UC or healthy controls [141]. It is interesting then, that in our study, the reduction in IFN-γ have an impact in the tissue damage observed. This effect was primarily observed in the ileum and cecum, and few differences in pathology were observed in the colon when comparing AIEC with the PBS-treated group.

Reduced inflammation in the absence of IbeA, due to *E. coli*-impaired ability to cross a biological barrier has been reported previously in NMEC. In an infant rat model, the strain RS218 lacking IbeA did not show a decreased level of bacteremia in the blood; however, the ability of RS218 to develop meningitis was drastically impaired (reduction of at least 40% [59]). This suggested that there was an impaired ability to cross the blood-brain barrier by the NMEC *ibeA* mutant and therefore cause inflammation inside the brain. In our model, to access the underlying immune cells in the GI tract and generate inflammation, AIEC needs to cross the intestinal barrier. The method we used to collect the organs does not discriminate between intracellular and extracellular bacteria, so it is possible that even when similar levels of wt and mutant bacteria were present throughout the course of the experiment, the *ibeA* mutant did have less access to the underlying immune cells in the host, and therefore caused less inflammation, as reflected in less IFN-γ production and reduced pathological score.

Regarding the inflammatory response observed, there was a correlation between colonization by AIEC and development of inflammation. A similar inflammatory response has been described in mice expressing CEACAM6 [140], as well as conventional mouse strains [98]. It is important to clarify that in conventional mouse strains, the hosts have a depleted microflora due to streptomycin treatment, and the introduction of a high dose (10^9) of AIEC might contribute to the inability of the microbiota to fully re-populate the intestine [137]. This might be one of the factors associated with the damage and the high pathology score compared to the PBS control. While in general terms, colonization of the gut by bacteria can elicit an inflammatory response, the fact that the response was observed up to 14 days post infection, suggests long-lasting damage to the gut even in the absence of bacteria. It is also possible that in the absence of IbeA, reduced localization to the Peyer's patches (M cells) occurs, and account for the trend in reduction of IFN-γ levels in the cecum and reduced histopathology observed in the ileum and cecum. In conclusion, our study has generated novel information regarding the mechanisms that AIEC have to interact with the host,

and adds another virulence determinant to the AIEC arsenal that might be contributing to the perpetuation of inflammation [137, 142].

CONCLUSIONS AND FUTURE DIRECTIONS

The goal of the research reported here was to define the role of the invasin IbeA in the interaction of AIEC with intestinal epithelial cells and macrophages. To achieve the proposed goals, an AIEC Δ*ibeA* mutant was generated, and the effect that this deletion had on AIEC invasion and adhesion was evaluated in Caco-2 cells as a model for enterocytes as well as in M cells. Different parameters were also evaluated in the adhesion and invasion process of AIEC to enterocytes and M cells, such as the effects of time and quantity of bacteria. Furthermore, the effect of enterocyte polarization on AIEC invasion was also evaluated. The results obtained showed consistently, that in the absence of IbeA, the invasion process of AIEC was dramatically affected (more than 50% reduction), in polarized enterocytes, non-polarized enterocytes, and M cells.

Not only was AIEC invasion of the intestinal epithelium affected, but transcytosis through M cells was also impaired in the absence of IbeA. These results could suggest that AIEC transcytosis through M cells might not necessarily be a passive mechanism, such as endocytosis or macropinocytosis, but it could be receptor-mediated endocytosis. AIEC transcytosis through M cells mediated by IbeA is mechanism in addition to the one already described (Lpf-mediated [50]) by which AIEC can access the underlying gut-associated immune cells and subsequently contribute to inflammation. Going forward, it would be important to determine the presence of AIEC *in vivo* or *ex vivo* in Peyer's patches (PP) localized the small intestine of mice. This proposed study would be useful to replicate the *in vitro* findings of this study, since in the absence of IbeA; reduced localization of AIEC within PP would be expected. As mentioned above, access to the underlying gut-associated immune cells is a critical step for the development of AIEC-associated inflammation.

This work becomes the first report of IbeA as an AIEC invasin; thus, there are many areas that require further exploration. I believe one of the most interesting areas to develop is a study of the prevalence of *ibeRAT* in a larger pool of AIEC isolates. The presence of *ibeA* together with other AIEC-associated virulence factors could be used as a combination to determine, upon genetic analysis, whether an *E. coli* strain belongs to the AIEC pathotype. This, in turn, would serve as a predictor of the invasiveness of these isolates, since not all IBD-associated *E. coli* isolates present the same AIEC phenotype. It is critical to develop complementary assays to facilitate the identification and classification of *E. coli* from IBD patients as AIEC, since currently their classification is solely based on phenotypic observations, which can be extensively time consuming and variable between laboratories. Furthermore, in this study for the first time, we have shown that IbeA is not only limited to *E. coli* associated with extraintestinal infections, but can play an important role in the interaction with the intestinal epithelium, such as in the case of AIEC, and atypical EPEC where IbeA might mediate the observed invasive phenotype.

Another important area that requires further study is the mechanisms behind AIEC invasion of intestinal epithelial cells, primarily which host cell components are used by AIEC IbeA to invade the GI tract. This area has not been explored, and, as mentioned above, it is likely that the mechanisms might differ from those following NMEC IbeA invasion of the brain endothelium.

Additionally, I have found that differences of AIEC colonization of the mouse GI tract *in vivo* were not observed in the absence of IbeA. In this *in vivo* study it was difficult to replicate the consistent invasive reduction observed *in vitro*. It is possible that one limitation of the *in vivo* model is the difficulty to discern between adherent and intracellular bacteria. To further validate the results described *in vitro*, it would be

important to adapt our *in vivo* assay, possibly by the incubation of the extracted organs with an antibiotic that selectively kills extracellular bacteria (similar to the role of gentamicin for *in vitro* assays), therefore allowing discrimination between intracellular AIEC and AIEC adhering to the intestinal epithelium. Other *in vitro* alternatives to epithelial cell mono-cultures should also be explored such as enteroid cultures. These *ex vivo* 3D cultures, containing multiple cell types of a normal intestinal epithelium within the same system, have been recently developed and might provide more extensive information of the role of IbeA in AIEC invasion in a complex system mimicking that of the human intestine [143], and serve as an alternative to *in vivo* studies. Overall, in this study, I have been able to show that IbeA, never described before in the AIEC pathotype, is at least one of the important players needed to establish the invasive phenotype observed in these CD-associated *E. coli*, and, furthermore, contributes to AIEC pathology by enabling these bacteria to cross an important biological barrier, the GI tract.

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