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Generation of enteropathogenic *E. coli* strains lacking the repertoire of effectors translocated by the type III protein secretion system and their characterization in the infection of cultured cell lines and human intestinal biopsies

Doctoral Thesis

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Tesis presentada por Massiel Cepeda Molero para optar al grado de Doctora en Ciencias por la Universidad Autónoma de Madrid.

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A Madrid

Miente que la tarde será eterna.

Esclaviza éste corazón a tu capricho
Martirio de cálido invierno y frío verano
Arrópalo en la calma de tu idilio,
Fugitivo y exiliado

Mátame un poco viviendo contigo cada día
Consiente mi alma con tu engaño
Dibuja con tu voz mi nombre por tus labios
Considera tu realidad mi fantasía.

Así que anda… miente un poco y dime que ésta tarde, será eterna…

A. Rojas

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ABREVIATIONS

A/E	Attachment and effacement
Amp	Ampicillin
AqA	Aquaporins
ARP2/3	Acting nucleating protein 2/3
BFP	Bundle-forming pilus
BL-1	Bax inhibitor-1
Bla	β-talactamase
BSA	Bovine serum albumin
CD	Crohn´s disease
CFU	Colony-forming unit
Cm	Chloramphenicol
CR	*Citrobacter rodentium*
CTB	B subunit of cholera toxin
DMEM	Dulbecco's Modified Eagle's médium
DSB	Double-strand brake
EAEC	Enteroaggregative *E. coli*
EAF	EPEC adherence factor
ECP	E. coli common pilus
Efa1	EHEC factor for adherence
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ETgA	Lytic transglycosilase
Fas	Focal adhesions
FRT	Flippase Recognition Target
GAPs	GTPase activating proteins
GDIs	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
GlcNAc	N-acetylglucosamine
GrlA	Global regulator of LEE activator
GrlR	Global activator of LEE repressor
GTP	Guanosine-5'-triphosphate
H-NS	Histone-like nucleoid-structuring protein
HRs	Homology regions
HUS	Hemolytic-uremic syndrome

IE	Integrative element
IHF	integrative host factor
IKK	IκB kinase
IL	Interleukin
IM	Inner membrane
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVOC	In vitro organ culture
Km	Kanamycin
LA	Localized adherence
LAB	Lactic acid bacteria
LB	Lysogenic broth
LCTs	Large clostridial toxins
LDH	Lactate dehydrogenase
LEE	Locus of enterocyte effacement
LifA	Lymphocyte inhibitory factor
LisM	Lysin motif
MAPK	Mitogen-activated protein kinase
MCS	Multiple cloning site
N-WASP	Wiskott-Aldrich Syndrome Protein
NcK	Host adaptor protein
NF-κB	Nuclear factor-κB
NHE3	Sodium hydrogen exchanger
NHEFR1	Hydrogen exchanger regulatory factor 1
Nle	Non-LEE effector
NLRP3	Nod-like Receptor 3
WIP	WASP interacting protein
OM	Outer membrane
OmpA	Outer membrane protein A
PAI	Pathogenicity island
PAMPs	Pathogens-associated molecular patterns
PerC	Plasmid-encoded-regulator C
PG	Petidoglycan
PI3K	Phosphatidylinositol-3 kinase
pIVOC	Polarized IVOC system
PPs	Prophages
PRRs	Polarized IVOC system
QS	Quorum sensing
RBS	Ribosome-binding site
RhoGEF	Rho guanine nucleotide exchange factor

RPS3	Ribosomal protein S3
SEM	Scanning electron microscopy
SGLT-1	Sodium-D-glucose transporter
STEC	Shiga toxin-producing *E. coli*
Stx	Shiga Toxins
T3SS	Type III secretion system
Tc	Tetracycline
Tir	Translocate Intimin Receptor
TJ	Tight junction
TNF-α	Tumor necrosis factor alpha
TRADD	TNF receptor type 1-associated DEATH domain protein
TRAF6	TNF receptor-associated factor 6
TRITC	Tetramethylrhodamine
UC	Ulcerative colitis
UPEC	Uropathogenic *E. coli*
UTIs	Urinary tract infection

SUMMARY

Although most *Escherichia coli* isolates are harmless commensals of the gastrointestinal tract, some strains have acquired specific virulence factors, like pathogenicity islands, insertion elements (IEs), and prophages (PPs), to become highly adapted pathogens. The enteropathogenic *E. coli* (EPEC) is an important category of diarrheagenic bacteria causing acute and chronic diarrhea in infants. The hallmark of EPEC infection is the formation of attachment and effacement (A/E) lesion in the intestinal mucosa surface, which is characterized by the intimate attachment of the bacteria to the enterocyte, microvilli effacement, and the formation of actin-pedestal-like structures underneath the attached bacteria. EPEC is endowed of a 35 kb pathogenicity island called the locus of enterocyte effacement (LEE) that contains all the genes necessary for the assembly of a type III secretion system (T3SS) injectisome. Through these injectisome EPEC translocates multiple effector proteins into the host cell to subvert cellular functions in benefit of the infection. The prototype strain E2348/69 of EPEC O127:H6 is endowed of six LEE encoded effectors and 17 non-LEE encoded effectors. We have engineered a set of effector mutant EPEC strains using suicide vectors to delete the whole repertoire of effector genes of this prototype EPEC strain. Genome manipulation did not affect the functionality of the T3SS injectisome. The deletion strategy was based on suicide or termosensitive plasmid integration by homologous recombination and the markerless resolution of co-integrants after I-*SceI* digestion. We did markerless integration of *map*, *espH* and *nleC* in their original locus in EPEC2 (maintain EspZ and Tir), EPEC1 (maintaining only Tir) and EPEC0 (effector-less) mutant strains. These strains were able to translocate functional effectors from chromosomal expression into HeLa cells. We infected intestinal human biopsies with the effector mutant EPEC strains to identify the effectors necessary for the induction of the A/E lesion in human intestinal tissues. We found that while EPEC2 and EPEC1 mutant strains were able to induce the actin-pedestal formation in HeLa cells *in vitro*, none of the biopsies infected with these strains had A/E lesion. These results demonstrated that effectors besides Tir and EspZ are essential to induce the A/E lesion formation in intestinal biopsies. We infected intestinal biopsies with several effector mutant EPEC strains and we found that effectors located outside the LEE are essential to induce efficient A/E lesion on human intestinal biopsies. Additionally we found that non-LEE effectors are characterized by having an additive effect to allow the A/E lesion development in these intestinal surfaces and that Efa1/LifA homologous proteins seem to play a major role in this process. Our results with intestinal biopsies strongly suggest that non-LEE effectors are necessary for the efficient formation of A/E lesion in the *in vivo* situation.

RESUMEN

Aunque la mayoría de los aislados de *Escherichia coli* son comensales del tracto gastrointestinal, algunas cepas han adquirido factores de virulencia específicos, como islas de patogenicidad, elementos integrativos (IEs), y profagos (PPs), para convertirse en patógenos altamente adaptados. La cepas de *Escherichia coli* enteropatógena (EPEC) son una categoría importante de bacterias productoras de diarrea aguda y crónica en niños de corta edad. La característica distintiva de la infección por EPEC es la formación de la lesión llamada de unión y borrado A/E (*Attaching and Effacing lesion*), que se caracteriza por una unión intima de la bacteria a los enterocitos, la destrucción de las microvellosidades, y la formación de estructuras en forma de pedestales debajo de las bacterias unidas. EPEC está dotada de una isla de patogenicidad de 35 kb llamada LEE (locus of enterocyte effacement) que contiene todos los genes necesarios para ensamblar los inyectisomas del sistema de secreción tipo III (T3SS). A través de estos inyectisomas EPEC transloca proteínas efectoras a la célula huésped para manipular diversas funciones celulares en beneficio de la infección. La cepa prototipo E2348/69 de EPEC O127:H6 tiene seis efectores codificados en la isla LEE y 17 efectores codificados fuera de la isla LEE, llamados genéricamente efectores no-LEE. Hemos construido un grupo de cepas mutantes en efectores de EPEC, utilizando vectores suicidadas para delecionar el repertorio de efectores de la cepa prototipo. La manipulación genómica no afectó la funcionalidad de los inyectisomas del T3SS. La estrategia seguida se basa en el uso de plásmidos suicidas y termosensibles que se integran por recombinación homóloga, seguida de una resolución de los co-integrantes tras digestión con I-*SceI,* produciendo deleciones libres de marcadores. También hemos realizado una integración libre de marcas de los genes *map*, *espH* y *nleC* en su sitio original en el cromosoma de las cepas mutantes EPEC2 (mantiene EspZ y Tir), EPEC1 (mantiene Tir) y EPEC0 (sin efectores). Las cepas generadas son capaces de translocar desde su expresión cromosómica los efectores individuales de forma funcional a células HeLa. Infectamos biopsias intestinales humanas con las cepas de EPEC mutantes en efectores para identificar los efectores necesarios para inducir la formación de la lesión A/E en tejidos intestinales humanos. Identificamos que mientras las cepas mutantes EPEC2 y EPEC1 inducen la formación de pedestales de actina durante la infección *in vitro* de células HeLa, ninguna de las biopsias infectadas por estas cepas presentó lesiones A/E. Estos resultados demuestran que otros efectores además de Tir y EspZ son esenciales para inducir la formación de la lesión A/E en las biopsias intestinales. Infectamos biopsias intestinales con varias cepas de EPEC mutantes en efectores y descubrimos que los efectores localizados fuera de la isla LEE son esenciales para inducir eficientemente la lesión A/E en las biopsias intestinales humanas. Además demostramos que los efectores no-LEE

se caracterizan por tener un efecto aditivo para permitir el desarrollo de la lesión A/E en estas superficies intestinales y que las proteínas homologas a Efa1/LifA parecen jugar un papel principal en este proceso. Nuestros resultados con biopsias intestinales apoyan un papel de los efectores no-LEE en la formación eficiente de la lesión A/E en la situación *in vivo*.

INTRODUCTION

1. Importance of the human-microbiota interaction

Humans appeared in the evolutionary scene of the Earth 3.5 billions years after microorganisms. Bacteria were necessary to make the Earth habitable; they put the first molecular oxygen in the Earth's atmosphere, creating the ozone layer and the conditions that permitted the later evolution of oxygen-utilizing creatures such as humans. Microorganisms are spread in all parts of Earth. They live in the artic ice, in the subsurface of the land, in the air and also in hot springs (Abigail, Salyer et al. 2002). Microorganisms are also present in humans, establishing a large and dynamic collection of microbes called microbiota, which colonizes body surfaces covered by epithelial cells exposed to the external environment like gastrointestinal tract, respiratory tract, skin and vagina (Tlaskalova-Hogenova, Stepankova et al. 2004). Microbiota is central for maintaining a healthy environment and giving some metabolic benefits and acting as a barrier against pathogens (Mai 2004, Bull and Plummer 2014). The number of bacteria present in mucosal surfaces and skin is similar or even exceeds the number of cells that conform the human body (Sender, Fuchs et al. 2016). The majority of these microbial cells representing the 70% are localized in big conglomerates of microbes in the intestinal gut, thereby the impact of these microbes in human physiology is likely to be more pronounced in the intestine. In the proximal and middle small intestine the microbial density is relatively low but it increase in the distal small intestine to 10^8 bacteria/ml of luminal content and in the colon it increase to 10^{12}/gram (Hooper, Midtvedt et al. 2002). The combined microbiome has been studied by metagenomic approaches revealing that it exceeds the complexity of the human genome (Mai 2009). By recruiting these resident microbes with metabolic capabilities the host is relieved of the need to evolve such functions. Through its immense metabolic capabilities, the gut microbiota contributes to human physiology by transforming complex nutrients, like dietary fiber into simple sugars and other nutrients that can be absorbed and otherwise the host will lose this energy source (Hooper, Midtvedt et al. 2002).

The main interaction between host immune system and the external environment is the intestinal epithelium surface, this continuous and dynamic interaction is important for the development and maturation of the host immune system (Bull and Plummer 2014, Landman and Quevrain 2015). In the gut many bacteria produce antimicrobial compounds and compete for nutrients and sites of attachment, thereby preventing colonization by pathogens, this is known as competitive-exclusion effect (Bull and Plummer 2014). Imbalance of gut microbial populations has important functional consequences and is implicated in chronic

gastrointestinal diseases such as Irritable Bowel Syndrome (IBS) and Inflammatory Bowel Disease (IBD)(Mai 2009). The use of high-throughput sequencing has allowed the identification of a great number of bacteria including uncultivable bacteria, with these techniques 395 bacterial phyla has been identified in the human gut to date. Most of the inferred organisms were anaerobes members of the *Firmicutes* and *Bacteroidetes* phyla and *Proteobacteria* (including *Escherichia coli*) and *Actinobacteria* phyla are found in small proportion, which is no surprising for anaerobes facultative species in the strict anaerobic environment of the colon (Eckburg, Bik et al. 2005).

The integrity of the epithelial gastrointestinal tract is an important defense against bacterial pathogens, thus there is high rate of epithelial cell turn over to maintain the gastrointestinal epithelial integrity (Kim, Ashida et al. 2010). Throughout the gastrointestinal tract there are also specialized secretory cells responsible to produce a mucus layer of mucin glycoproteins and antimicrobial compounds to protect the epithelial surface from commensal bacteria of the microbiota and specialized bacterial pathogens (Ribet and Cossart 2015). Resident microbiota is important to induce mucus biosynthesis, the cell turnover and the formation of tight junctions (TJs) between cells (Kim, Ashida et al. 2010, Ribet and Cossart 2015). Commensal bacteria do not generally produce disease but in certain conditions like mucosal surface damage, they can become opportunistic pathogens and produce disease (McGuckin, Linden et al. 2011). Interestingly bacterial pathogens have acquired mechanisms to cross host barriers and produce disease despite the defense mechanisms of the host (Kim, Ashida et al. 2010).

2. Enteric bacterial pathogens

Enteric bacterial pathogens have evolved virulence traits that enable them to colonize the intestinal tract, adhere to or efface the epithelium, deliver enterotoxins and invade intestinal epithelial cells. Using these mechanisms bacterial pathogens can disrupt intestinal functions and cause malabsorption or diarrhea and in some cases cross the intestinal epithelial barrier and get deeper tissues and induce systemic disease (Guerrant, Steiner et al. 1999). The bacteria pathogens of the genus *Salmonella*, *Shigella* and *Listeria* are enteric pathogens that can invade the cells lining the surface of the intestine and replicate inside them and later disseminate in the host (Mastroeni and Grant 2011, Ribet and Cossart 2015). Most *Escherichia coli* strains are harmless and commensal inhabitants of the gastrointestinal tract. Acquiring specific virulence factors, like pathogenicity island (PAI), prophages (PPs) and plasmids, certain isolates may become highly adapted pathogens able to

induce a range of diseases, from gastroenteritis to extraintestinal infections of the urinary tract, bloodstream and central nervous system (Croxen and Finlay 2010). Urinary tract infections (UTIs) are the most common extraintestinal infections produce by the so-called uropathogenic *E. coli* (UPEC) strains. Among the intestinal pathotypes of *E. coli*, which are able to cause disease in healthy human, there are the noninvasive enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EAEC) and also there is an invasive pathotype, which is able to replicate inside epithelial cells, named enteroinvasive *E. coli* (EIEC) (Kaper, Nataro et al. 2004).

3. Enteropathogenic *Escherichia coli* (EPEC)

Enteropathogenic *E. coli* (EPEC) was the first pathotype of *E. coli* to be associated with human disease, it is an important category of diarrheagenic bacteria causing acute and chronic diarrhea in infants (Kaper, Nataro et al. 2004). The low microbial density of the small bowel caused by the forceful peristalsis in this part of the intestine is overcome by EPEC, which can successfully colonize the small intestine of humans (Hicks, Frankel et al. 1998, Tlaskalova-Hogenova, Stepankova et al. 2004). EPEC primarily affects children younger than 2 years old, however some outbreaks of EPEC infection in healthy adults have been associated with large inoculum ingestion (Nataro and Kaper 1998). The mechanism of transmission of EPEC is the fecal-oral route, with contaminated hands, water, food or fomites serving as vehicles (Nataro and Kaper 1998). The reservoirs of EPEC infection are symptomatic and asymptomatic children and asymptomatic adults carriers, who handle young children (Nataro and Kaper 1998). In the 1940s and 1950s EPEC was an important cause of diarrhea in developed countries with a 50% of mortality during outbreaks, but now the infection by EPEC in developed countries has a limited importance. In contrast, in low-income countries, EPEC is still an important cause infant diarrhea, representing between the 30% and 40% of the bacterial diarrheal pathogens in countries of South Africa and South America (Nataro and Kaper 1998). EPEC strains include a diverse group of serotypes that are divided into typical (tEPEC) and atypical (aEPEC) strains based of the presence or the absence of a large virulence plasmid called EAF (EPEC adherence factor). In low-income countries tEPEC are the leading cause of infantile diarrhea while aEPEC seems to be the more important cause of diarrhea in developed countries (Trabulsi, Keller et al. 2002).

3.1 EPEC pathogenesis

The hallmark that defines EPEC infection is the Attachment and Effacement (A/E) lesion (Garmendia, Frankel et al. 2005). By adhering to intestinal epithelial cells, EPEC subvert cytoskeletal processes of the cell to produce a histopathological feature known as the A/E lesion. This lesion is characterized by the intimate attachment of the bacteria to the intestinal epithelial cells, induction of cytoskeletal changes including the accumulation of polymerized F-actin in pedestal-like structures underneath the attached bacteria, and microvilli surface disruption (effacement) (Kaper, Nataro et al. 2004). The A/E lesion is also characterized by microcolony formation and microvilli elongation in the area surrounding the microcolony (Knutton, Lloyd et al. 1987)(Figure 1). EPEC together with EHEC and *Citrobacter rodentium* (CR) are members of the A/E family since they colonize the gastrointestinal tract via the A/E lesion. EPEC and EHEC are important human pathogens while CR is a mouse-restricted pathogen (Mundy, Girard et al. 2006). The main difference between EPEC and EHEC pathogenesis is that EHEC produces enterotoxins. EHEC belongs to a group of pathogenic bacteria known as Shiga toxin-producing *E. coli* (STEC), which are define by their ability to produce Shiga Toxins (Stx). EHEC infection induces bloody diarrhea and the most serious complication of EHEC infection is the hemolytic-uremic syndrome (HUS). HUS is a microangiopathic hemolytic anemia characterized by disseminated capillary thrombosis and ischemic necrosis, which principally affect kidneys producing renal failure although it can affect other organs (Donnenberg and Whittam 2001).

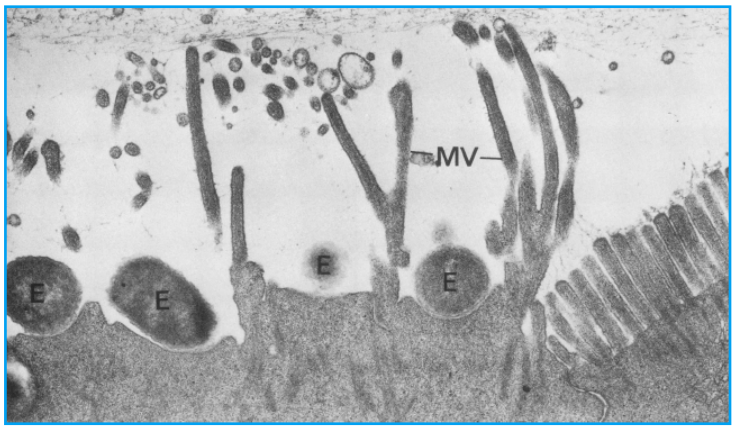

Figure 1. A/E lesion induced by EPEC in human duodenal biopsy. Bacteria are attached in the enterocyte surface in pedestal like structures. See the microvilli effacement at the site of bacterial attachment. The microvilli are elongated in the edge of the microcolony. Transmission electron micrograph adapted from Knutton et al., 1987

4. Key genetic and structural traits for A/E lesion formation

4.1 Locus of enterocyte effacement (LEE)

The ability of EPEC to induce A/E lesion is related with a pathogenicity island (PAI) of 35-kb called the locus of enterocyte effacement (LEE) (McDaniel, Jarvis et al. 1995). Homologues of LEE are also found in the other members of the A/E pathogens family EHEC and CR (Frankel, Phillips et al. 1998). The G+C content of the LEE is 38%, which is lower than the 51% of G+C content of the total *E. coli* genome and this indicates horizontal gene transfer of this pathogenicity island into *E. coli* from another species (Frankel, Phillips et al. 1998). The LEE comprises 41 genes organized in five principal operons LEE1-LEE5 and several smaller transcriptional units (Figure 2) (Yerushalmi, Litvak et al. 2014). The LEE encodes all the structural genes necessary for the assembly of a type III secretion system (T3SS) injectisome on the bacterial cell envelope (McDaniel and Kaper 1997). The LEE also encodes transcriptional regulators (Ler, GrlR and GrlA), translocator proteins (EspA, EspB and EspD), six secreted effectors proteins (including the Translocated Intimin Receptor Tir), the outer membrane protein Intimin, molecular chaperones and a lytic transglycosilase (EtgA) (Dean, Maresca et al. 2005). The muramidase activity of the EtgA has been reported to enhance the levels of T3SS injectisome (Garcia-Gomez, Espinosa et al. 2011). EtgA interacts with the injectisome component EscI to locally hydrolyse the peptidoglycan (PG) during assembly (Burkinshaw, Deng et al. 2015)

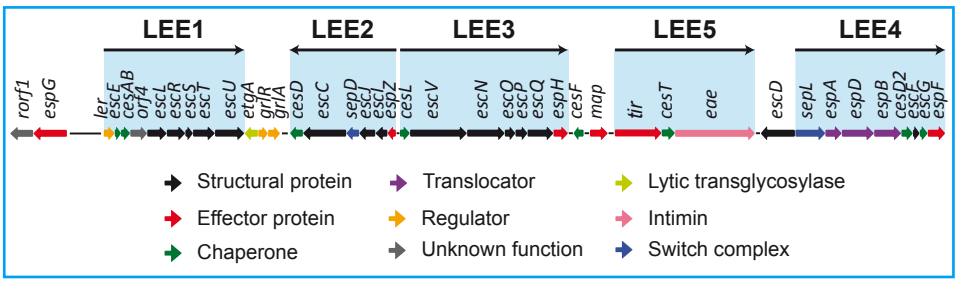

Figure 2. The Locus of enterocyte effacement (LEE) in EPEC. The LEE is constituted by five principal operons LEE1-LEE5 and other smaller transcriptional units. The LEE encodes all the structural proteins for the assembly of the T3SS injectisome, regulators for LEE expression, chaperones proteins and six effectors proteins.

The mechanism of regulation of LEE is complex and depends on environmental conditions, quorum sensing (QS) and in several transcriptional regulators encoded in the LEE and outside the LEE (Garmendia, Frankel et al. 2005). The histone-like nucleoid-structuring protein (H-NS) is responsible of silencing the LEE operons during repressive conditions (Jobichen, Li et al. 2007). The first gene in LEE1 is

ler, encoding a positive regulator for all other LEE promoters; Ler functions as anti H-NS, to alleviate the H-NS-mediated repression (Yerushalmi, Litvak et al. 2014). The expression of *ler*, and thus of LEE genes, is regulated both negatively and positively by a complex myriad of global regulatory proteins in response to different environmental signals (Bustamante, Villalba et al. 2011) . The GrlA (global regulator of LEE activator) and GrlR (Global activator of LEE repressor), encoded by the *grlRA* operon within the LEE are involved in positive and negative regulation *ler* expression, respectively (Garmendia, Frankel et al. 2005). Other positive regulators of *ler* expression like PerC (Plasmid-encoded-regulator C) and IHF (integrative host factor) overlaps with the GrlA activation depending on the growth conditions (Bustamante, Villalba et al. 2011). Ler also regulates the expression of genes encoded outside the LEE like *espC* and non-LEE-effectors, such as *nleA* (Kaper, Nataro et al. 2004, Garcia-Angulo, Martinez-Santos et al. 2012).

4.2 The EPEC adherence factor (EAF) plasmid pMAR2

The prototypical EPEC strains E2348/69 is endowed of a 97kb plasmid called EAF for EPEC adherence factor, or pMAR2 (Iguchi, Thomson et al. 2009). The pMAR2 contains the operon *perABC* (plasmid encoded regulator). PerC regulates *ler* expression whereas PerA activates the expression of *bfp*, a 14-gene operon encoding for the bundle-forming pilus (BFP), a rope-like bundle of type IV pili (Stone, Zhang et al. 1996). BFP allows EPEC bacteria to form microcolonies in a pattern called localized adherence (LA) and also mediates the initial interaction of bacteria with host cells surfaces (Brinkley, Burland et al. 2006, Hyland, Sun et al. 2008).

4.3 The Type III secretion system

The type III secretion system (T3SS) is a macromolecular transport apparatus that is used by many gram-negative bacteria (e.g. *Shigella*, *Yersinia*, *Salmonella*) to translocate virulence proteins, also called effectors, into the cytosol of infected cells, subverting host cellular functions in profit of the infection (Ogino, Ohno et al. 2006). Since pathogens use this transport apparatus to inject proteins, this structure is also known as the injectisome. T3SS is closely related to the flagellum, which have given useful information about the structure and functionality of the T3SS (Erhardt, Namba et al. 2010). EPEC T3SS mediates the translocation of multiple effector protein during infection. Some of them are encoded in the LEE whereas others are encoded outside of the LEE being generally referred to as non-LEE effectors (Nle) (Garmendia, Frankel et al. 2005). The T3SS of EPEC is

assembled for more than 20 proteins. This molecular nanomachine is composed of three portions: (i) the multiring basal body, which functions as a channel that spans the outer and inner membranes as well as the periplasmic space and houses the export apparatus, (ii) a hollow extracellular filamentous structure that forms a conduit to the host cell and (iii) a translocation pore formed in the host cell membrane by proteins known as translocators (Figure 3).

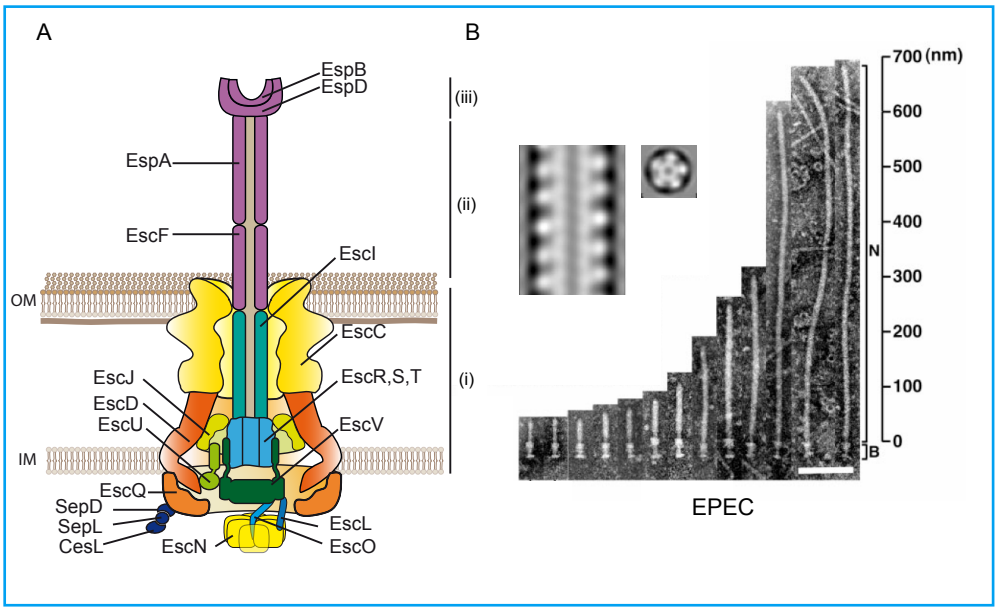

Figure 3. Composition of the T3SS injectisome of EPEC. A. Schematic representation of the injectisome with the basal body (i), the extracellular filamentous structure (ii) and the translocation pore (iii). B. Electron micrograph of purified T3SS of EPEC showing that EspA filament is more than 600 nm long and 3D reconstruction of the EspA filament, showing the internal channel. Figures are adapted from Sekiya et al., 2001 and Daniell et al., 2003

The basal body is formed by a set of ring-like protein structures; the EscC, which forms the outer membrane (OM) ring, belongs to the members of the secretin superfamily that participates in the delivery of large molecules through the OM (Ogino, Ohno et al. 2006, Galan, Lara-Tejero et al. 2014). The EscD and the lipoprotein EscJ are forming the inner membrane (IM) ring. The EscI polymerizes to assemble the periplasmic inner rod of the basal body (Ogino, Ohno et al. 2006). The basal body has five proteins associated to the IM (EscR, EscS, EscT, EscU and EscV) that constitute the export apparatus (Romo-Castillo, Andrade et al. 2014). Associated to the basal body there is cytoplasmic ring formed by EscQ, that recruits the ATPase of the system called EscN, which is necessary to energize the protein translocation (Biemans-Oldehinkel, Sal-Man et al. 2011). The hollow filamentous needle structure is formed by polymerization of EscF to assemble the needle with an inner diameter of 2-3 nm, which is thought to allow the translocation of unfolded

proteins (Blocker, Jouihri et al. 2001, Wilson, Shaw et al. 2001). Attached to the tip of the needle is the EspA filament, that is a helical tube of polymerized EspA, with more than 600 nm of length and central channel of 2.5 nm of diameter (Sekiya, Ohishi et al. 2001, Daniell, Kocsis et al. 2003). EspA enables the translocator proteins EspB and EspD to insert into the host cell membrane and assemble a pore of 3-5 nm to allow translocation of effector proteins into the cell (Ide, Laarmann et al. 2001, Luo and Donnenberg 2011).

Type III secreted proteins, including translocator and effector proteins, have two dedicated domains recognized by the T3SS machinery: the N-terminal signal, spanning the first ~20 amino acid residues of these polypeptides, and the chaperone-binding domain located downstream from the N-terminal signal (Ghosh 2004, Arnold, Brandmaier et al. 2009, Munera, Crepin et al. 2010). These chaperones are essential to ensure efficient secretion and translocation of T3-secreted proteins; CesAB, CesD and CesD2 are the corresponding chaperones of the EspA, EspB and EspD translocator proteins whereas CesT and CesF are the chaperones of effector proteins (Garmendia, Frankel et al. 2005, Thomas, Deng et al. 2005). The system has also two essential chaperones EscE and EscG that prevent premature polymerization of the EscF needle protein in the cytosol of the bacteria (Sal-Man, Setiaputra et al. 2013).

The assembly of the T3SS is a finely regulated process that requires many cytoplasmic and membrane associated proteins and chaperones (Monjaras Feria, Garcia-Gomez et al. 2012). At least two molecular switches participate in the assembly of functional injectisomes. EscU is an IM-associated protein with a cytoplasmic domain that is autocleaved, which induces a conformational change in EscU essential for the interaction with other T3SS components (Zarivach, Deng et al. 2008, Monjaras Feria, Garcia-Gomez et al. 2012). EscP, a weakly secreted protein of the T3SS, controls needle length by interacting with both EscF needle protein and EscI. EscP is suggested to sense when the needle is completed interacting with the cleaved cytoplasmic domain of EscU, inducing a conformation change in this protein that flicks a specificity switch to allow secretion of translocator (EspA, EspB, EspD) proteins by the export apparatus (Monjaras Feria, Garcia-Gomez et al. 2012). EscP also interacts with the chaperone CesT, which together with Tir and SepD-SepL complex, block secretion of effectors until the translocation pore is assembled in the host membrane (Monjaras Feria, Garcia-Gomez et al. 2012). The SepD-SepL complex along with CesL, the chaperone of SepL, sense an undefined environmental signal when the translocator pore is assembled and this induces a second switch from translocator to effector secretion (Deng, Li et al. 2005, Younis,

Bingle et al. 2010). This second switch allows the injection of effector proteins into the host cell. There is a hierarchy for the translocation of effectors, being Tir the first effector to be translocated in greater rate compared to the rest of effectors (Thomas, Deng et al. 2007, Mills, Baruch et al. 2013). Tir also influences the efficiency of injection of other effector proteins, likely due to the increased bacterial adhesion to host cells (Battle, Brady et al. 2014).

5. Model of EPEC pathogenesis

EPEC pathogenesis is described in a three-stage model that includes: (i) localized adherence to host cells, (ii) signal transduction and intimate attachment, and (iii) subversion of host cellular processes.

5.1 Localized adherence to host cells

EPEC is endowed of a tight regulation of its virulence genes in response to environmental conditions. This regulation is less metabolically expensive for EPEC and avoids alerting the human immune system of the presence of EPEC prior to a successful colonization of the small intestine (Clarke, Haigh et al. 2003). Environmental conditions such as temperature (Yerushalmi, Litvak et al. 2014), the increase of pH in the small intestine compare to the stomach (Fallingborg 1999, Shin, Castanie-Cornet et al. 2001), the auto-inducer molecule 3 and hormones epinephrine and norepinephrine, which are released by the host during stress conditions, induce expression of EPEC virulence factors (Franzin and Sircili 2015). These signals are sensed by EPEC and integrated with its specific regulator PerC and with global transcriptional regulators of *E. coli* like IHF and H-NS between others, to induce expression of EPEC virulence genes like the *bfp* operon and the *LEE*, which participates in the initial attachment to the small intestine.

At the beginning of the infection EPEC bacteria interact with the intestine-lining surface in a non-intimate manner. This contact is done mainly through the BFP that acts as an early adhesin in EPEC colonization of the human intestine. This initial adhesion is done by the interaction of the BFP with *N*-acetyl-lactosamine containing receptors in the host cell surface (Cleary, Lai et al. 2004, Hyland, Sun et al. 2008) (Figure 4). EspA also promote attachment to the enterocytes, albeit in a less efficient manner than the BFP (Cleary, Lai et al. 2004). In addition, flagella (Giron, Torres et al. 2002) and the *E. coli* common pilus (ECP) (Rendon, Saldana et al. 2007) and the lymphocyte inhibitory factor (*LifA*) (Badea, Doughty et al. 2003)

are also involved in this non-intimate interaction between the bacteria and the intestinal epithelium.

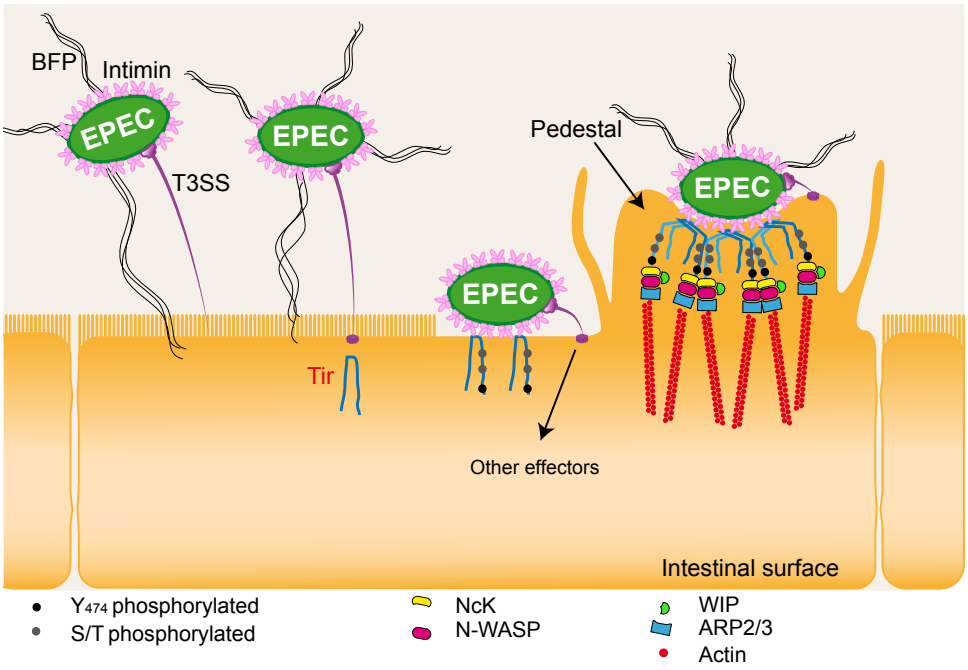

Figure 4. Localized adherence and intimate attachment of EPEC to the intestinal surface. At an early stage, EPEC interacts in a non-intimate manner with the intestinal surface mainly through the BFP and EspA filament. After assembly of the translocation pore, EPEC injects Tir. Ser/Thr phosphorylation of Tir induces its anchoring in the enterocyte plasma membrane, leaving TirM region exposed for the interaction with Intimin. Tir-Intimin interaction induces clustering and dimerization of Tir and this activates a signal cascade that generates actin polymerization and the pedestal formation underneath the attached bacterium and the intimate attachment of the bacteria to the enterocytes. Tir phosphorylation of residue Y474 engages the host adaptor NcK, which later recruits N-WASP and WIP. N-WASP recruits ARP2/3 complex, which induces actin nucleation and polymerization

5.2 Signal transduction and intimate attachment

Upon EPEC interaction with the enterocytes, EspB and EspD proteins are inserted into host cell membrane assembling the translocation pore (Guignot, Segura et al. 2015). The complete assembly of the T3SS allows the injection of EPEC own receptor, which is a 78 kDa protein called translocated intimin receptor (Tir) (Kenny, DeVinney et al. 1997). Following translocation, Tir is phosphorylated in serine/threonine residues in its C-terminus by host kinases and this induces conformational shifts promoting the insertion of Tir into the apical host cell membrane (Kenny 2002). This insertion leaves the N- and C-terminal portions of Tir exposed to the cytosol of the host cell with helical transmembrane domains

traversing the host apical membrane and the central portion of about 100 aa known as TirM displayed on the surface of the cell (Lai, Rosenshine et al. 2013). TirM domain contains two α helices separated by a hairpin loop and this conformation mediates the interaction with a 94 kDa OM adhesin protein of EPEC called Intimin (Luo, Frey et al. 2000). Intimin comprises an N-terminal signal peptide for IM secretion through the Sec-translocon, followed by a short periplasmic LysM-domain that binds the PG, a β-barrel domain that anchors the protein to the OM, and a extracellular segment composed by three immunoglobulin (Ig)-like domains and one C-terminal lectin-like domain, which mediates the interaction with TirM (Luo, Frey et al. 2000, Touze, Hayward et al. 2004, Leo, Oberhettinger et al. 2015). The C-terminal region of Intimin is also proposed to confer tissue specificity for A/E lesion formation in the small intestine by EPEC (Phillips and Frankel 2000, Reece, Simmons et al. 2001). Intimin forms homodimers in the OM mediated by the dimerization through the N-terminal region of periplasmic domains containing LysM (Leo, Oberhettinger et al. 2015). Intimin binding to Tir is proposed to induce clustering and dimerization of Tir in the host cell membrane, which is important to establish the intimate bacterial adherence and to initiate cellular signaling cascades for actin pedestal formation (Touze, Hayward et al. 2004, Lai, Rosenshine et al. 2013).

Tir interacts with host proteins through its N- and C-terminal cytosolic domains, the N-terminal domain of Tir interact with focal adhesion proteins in a tyrosine phosphorylation independent manner stabilizing Tir interaction with the host cytoskeleton (Goosney, DeVinney et al. 2001). On the other hand, tyrosine phosphorylation of the C-terminal region of Tir by host cellular protein kinases is required for efficient actin polymerization. Phosphorylation of residue Y474 is the most critical for focal actin assembly and is the major pathway to initiate pedestal formation (DeVinney, Puente et al. 2001). The pedestal formation requires a host adaptor protein called NcK, which contains and SH2 domain capable of binding a 12 residues region that includes the critical phosphorylated Y474 (Campellone, Giese et al. 2002). NcK also contains three SH3 domains that may directly recruits the neural Wiskott-Aldrich Syndrome Protein (N-WASP) or indirectly through the intermediary adaptor, WASP Interacting Protein (WIP) (Lai, Rosenshine et al. 2013, Young, Clements et al. 2014). The N-WASP protein successively recruits and activates the actin nucleating ARP2/3 complex, which induces the actin nucleation and polymerization. Actin polymerization drives membrane protrusion and pedestal formation (Kalman, Weiner et al. 1999). There are also minor NcK independent mechanisms of actin pedestal formation, indicating redundant pedestal formation pathways. Most of this activity also requires Tir Y474, reaffirming its role as a key

residue for triggering actin assembly by Tir. In the absence of Y474, residue Y454 of Tir is critical for this minor NcK independent actin assembly (Campellone and Leong 2005)(Figure 4).

5.3 Subversion of host cellular processes

Most EPEC strains remain extracellular in the small bowel during the infection and rely upon the T3SS to deliver effector proteins directly into the host cell to subvert numerous host cell functions (Donnenberg and Kaper 1992, Ochoa and Contreras 2011). Some of the effector proteins are encoded within the LEE: EspF, EspG, EspH, Map, EspZ, Tir, plus the translocator pore-forming protein EspB that also has effector functions in the cells (Garmendia, Frankel et al. 2005, Iizumi, Sagara et al. 2007). Also important are non-LEE encoded effectors (Nle), EPEC serotype O127:H6 strain E2348/69 has 17 Nle effectors genes localized in integrative elements (IEs) and prophages (PPs) distributed around EPEC genome (Iguchi, Thomson et al. 2009, Deng, Yu et al. 2012). The majority of these effector proteins are multifunctional and have the ability to work together in redundant, synergetic or antagonistic relationships over the host cells in benefit of EPEC infection(Dean, Maresca et al. 2005). EPEC E2348/69 is the prototype strain most widely used worldwide to study EPEC infection, although there are other EPEC serotypes and strains with different repertoires of T3SS effectors (Iguchi, Thomson et al. 2009).

5.3.1 Actin cytoskeletal manipulation

EPEC translocates effectors proteins that manipulate host actin cytoskeleton, in other to allow the progression of the infection (Alto, Shao et al. 2006, Wong, Raymond et al. 2012) (Figure 5). The effector protein Map belongs to a WxxxE family of bacterial effectors with the capacity to affect the Rho GTPase signaling pathways (Alto, Shao et al. 2006). Map functions as potent guanine-nucleotide exchange factor (GEF) for the Cdc42 Rho GTPase (Huang, Sutton et al. 2009). The activation of Cdc42 triggers a transient filopodia formation at bacterial attachment sites during early stages of EPEC infection (Berger, Crepin et al. 2009). Map also induces filopodia stabilization in a mechanism that involves indirect activation of the small Rho GTPase RhoA and RhoA-ROCK pathway (Berger, Crepin et al. 2009). The Map-mediated Cdc42 signaling inhibits Tir-Intimin triggered pedestal formation (Kenny 2002). Tir antagonizes the Map-induced filopodia formation, in mechanisms that involve Intimin-Tir interaction, either because this interaction is propose to release a C-terminal encoded GAP-like activity to down-regulated Cdc42 function by stimulating its conversion to the GDP-bound inactive form and/

or by the activation of Tir actin polymerization cascades (Kenny 2002, Kenny 2002, Berger, Crepin et al. 2009). The relevance of Map-mediated filopodia formation on cultured cells to *in vivo* infection is not yet known (Wong, Pearson et al. 2011). EspH also manipulates actin cytoskeleton of the infected cell. EspH down-regulates filopodia formation, while induces actin polymerization and pedestal elongation in an Nck-independent recruitment of WIP and N-WASP (Tu, Nisan et al. 2003, Wong, Raymond et al. 2012). Infection with EPEC overexpressing EspH indicates that EspH is a potent inducer of cytotoxicity and cell detachment. EspH induces disassembly of focal adhesions (FAs) concomitantly with actin disruption, generating a cell rounding phenotype (Wong, Clements et al. 2012). The multifunctional effector EspF also induces pedestal maturation, through the recruitment of junctional proteins into the pedestal (Peralta-Ramirez, Hernandez et al. 2008).

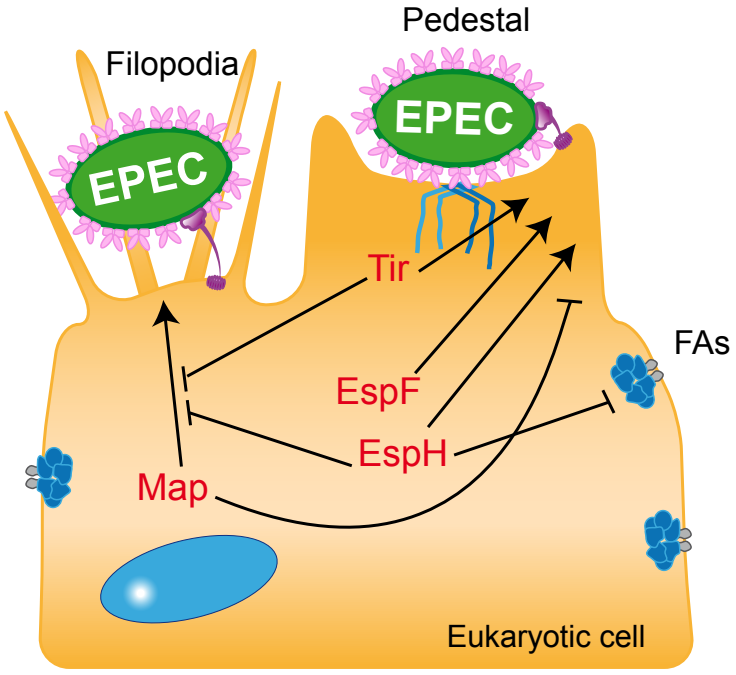

Figure 5. Actin manipulation by EPEC effector proteins. Map induces a transient filopodia formation at the beginning of the infection at the site of bacterial attachment and inhibits pedestal formation. Tir and EspH antagonize this filopodia formation and induce actin polymerization and pedestal formation. EspH induces disassembly of Focal Adhesions (FAs). EspF induces pedestal maturation.

5.3.2 Alterations of epithelial function

The intestinal epithelium acts as a physical barrier separating luminal environment and subepithelial tissues. The formation of intercellular TJs is important to maintain

this intestinal barrier function (Dean and Kenny 2004). The microvilli surface of the intestinal epithelium increases the absorptive area of the intestine and contains a broad of digestive enzymes, nutrient and electrolyte transporters (Lapointe, O'Connor et al. 2009). During EPEC infection the cytoskeletal rearrangement to induce pedestal formation results in microvilli destruction, which severely diminish the absorptive capacity of the infected intestinal surface, driving to loss of water and consequent diarrhea (Buret, Olson et al. 1998). The rapid onset of severe watery diarrhea prior to the full A/E lesion formation is suspected to be mediated by an active secretory mechanism in which T3SS effectors affect water and ion transport (Lapointe, O'Connor et al. 2009) (Figure 6).

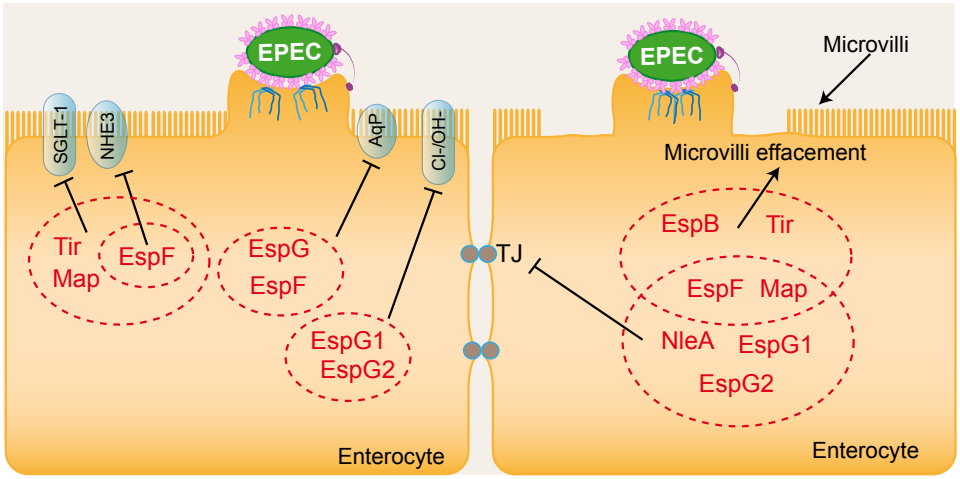

Figure 6. EPEC effector proteins altering epithelial cell function and inducing water loss and diarrhea. Tir, Map and EspF inhibit the sodium-D-glucose transporter (SGLT-1). EspF reduces expression of the sodium hydrogen exchanger (NHE3). EspG and EspF induce mislocalization of aquaporins (AqP). EspG1/EspG2 alter membrane targeting of Cl-/OH- exchanger. EspF, Map, NleA, EspG1 and EspG2 disrupt the tight junctions (TJ). EspB, Tir, EspF and Map induce microvilli effacement.

The cooperative action of Tir, Map and EspF inhibits the sodium-D-glucose transporter (SGLT-1), the major water pump of the small intestine, responsible for about 70% of the total fluid uptake (Dean, Maresca et al. 2006). The multifunctional effector EspF reduces expression of the sodium hydrogen exchanger (NHE3), which reduces the Na^+ absorption. It is also reported that Map through a TRL (Thr-Arg-Leu) motif is involved in a protein complex that include the NHEF1 (sodium hydrogen exchanger regulatory factor 1) and induce phosphorylation of the NHE3 leading to its inhibition (Simpson, Shaw et al. 2006). Since water osmotically moves towards areas of higher salt concentrations, changes in Na^+ absorption leads to

diarrhea (Hecht, Hodges et al. 2004, Hodges, Alto et al. 2008). EspG1 and EspG2 proteins alter the membrane targeting of Cl^-/OH^- exchanger (DRA), resulting in reduce Cl^- uptake and its accumulation in the lumen, which also drives water loss. This effect seems to be mediated by EspG/EspG2 disruption of microtubular network (Gill, Borthakur et al. 2007). EspF and EspG induce mislocalization of aquaporins (AQP), reducing the water absorption from the intestinal lumen (Guttman, Samji et al. 2007, Croxen and Finlay 2010). The translocator protein EspB interacts with myosin inhibiting the interaction of myosin-actin filaments and inducing microvillus effacement (Iizumi, Sagara et al. 2007). Tir, EspF and Map also induce microvillus effacement and this disruption of the brush border surface certainly exacerbate EPEC diarrhea (Dean, Maresca et al. 2006). Other effectors like EspF and Map act synergistically disrupting TJs, inducing mislocalization of the transmembrane TJs protein occludin (McNamara, Koutsouris et al. 2001, Dean and Kenny 2004). EspG1/EspG2-induced microtubule-disruption contributes to TJs disruption by a shift in localization of TJ proteins to the cytosol and the NleA effector protein also disrupts TJs blocking the delivery of new TJs proteins (Kim, Thanabalasuriar et al. 2007, Thanabalasuriar, Koutsouris et al. 2010, Glotfelty, Zahs et al. 2014). The TJs disruption done by these effectors increase intestinal permeability, and this could contribute to EPEC-induced diarrhea (Croxen, Law et al. 2013).

5.3.3 Subversion of host immune pathways

EPEC infection is characterized by a weak inflammatory response (Dean and Kenny 2009). Some LEE effectors proteins and a bigger group of Nle effectors overcome the host immune response, which favors bacterial survival (Figure 7).

5.3.3.1 Counteracting phagocytosis

Phagocytosis is a key mechanism of the innate immune system to fight against pathogens (Santos and Finlay 2015). Several effectors of EPEC counteract phagocytosis. EspB interfere with phagosome closure and prevent EPEC internalization in macrophages by interaction with myosin (Iizumi, Sagara et al. 2007). EspF prevents the phosphatidylinositol-3 kinase (PI3K) dependent F-actin rearrangement required for phagocytosis (Quitard, Dean et al. 2006). EspH binds mammalian Rho guanine nucleotide exchange factors (RhoGEFs) preventing actin reorganization necessary to induce phagocytosis. Lastly EspJ prevents opsono-phagocytosis (Marches, Covarelli et al. 2008, Dong, Liu et al. 2010).

5.3.3.2 Modulation of inflammatory signaling pathways

The PAMPs (pathogens-associated molecular patterns) are recognized by intestinal epithelial cells, through PRRs (pattern recognition receptors), this interaction triggers the activation of proinflammatory pathways such as; the nuclear factor-κB (NF-κB) and the mitogen-activated protein kinase (MAPK) (Takeuchi and Akira 2010). The NF-κB refers to transcription factor complexes, which include c-Rel, RelB, p65, p50, and p52. In unstimulated cells, homodimers or heterodimers of NF-κBs proteins (being p65/p50 the most abundant) are maintained in an inhibited state in the cytoplasm bound to IκB (Muhlen, Ruchaud-Sparagano et al. 2011). Following host cell detection of PAMPs by PRRs, a signaling cascade is initiated leading to activation IκB kinase (IKK) complex, which phosphorylates IκB targeting it for proteasome degradation (Liou 2002, Takeuchi and Akira 2010). IκB degradation frees p65/p50 allowing them to translocate to the nucleus, where they activate transcription of cytokine genes including IL-1β, IL-6, IL-8 and TNF-α (Pahl 1999).

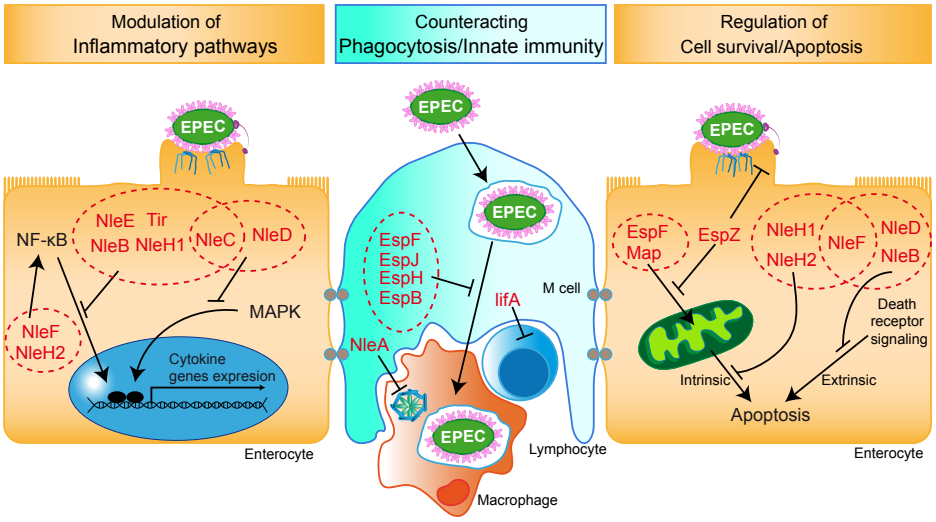

Figure 7. Schematic representation of multifunctional and overlapping effectors to control host immune response. NF-κB proinflammatory pathway is activated by NleF and NleH2 and is inhibited by NleE, NleB, NleH1, Tir and NleC. NleC and NleD inhibit the MAPK proinflammatory pathway. EspF, EspJ, EspH, EspB prevent macrophage phagocytosis. NleA disrupts inflammasome activation and LifA inhibits IL-2 and IL-4 production and lymphocyte proliferation. EspF and Map induce intrinsic apoptosis and EspZ counteracts these effects stabilizing mitochondrial membrane potential. NleH1/NleH2 and NleF counteract the intrinsic apoptosis and NleF, NleD and NleB counteract the extrinsic apoptosis.

It has been reported that during early stage of infection the injection of NleF by EPEC induces NF-κB activation and expression of IL-8 (Pallett, Berger et al. 2014). Also NleH2 induces NF-κB activation (Pham, Gao et al. 2012). Nevertheless, EPEC translocates several effectors that target the proinflammatory pathways of the cell, and this counteracts the proinflammatory effect of EPEC to allow progression of the infection (Wong, Pearson et al. 2011). Tir also interferes with the NF-κB signaling pathway; the interaction of Tir with SPH-2 cellular tyrosine phosphatase facilitates the recruitment of TNF receptor-associated factor (TRAF6) and inhibits the downstream cytokine production (Yan, Quan et al. 2013). NleE inhibits the TNF-α and IL-1β-induced activation of NF-κB by preventing the proteasome degradation of IκB. The NleE-mediated stabilization of IκB prevents p65 nucleation and the downstream cytokine expression (Newton, Pearson et al. 2010). NleB inhibits the TNF-α-induced activation of NF-κB also preventing the IκB degradation (Newton, Pearson et al. 2010). NleB has a N-acetylglucosamine (GlcNAc) transferase activity that specifically glycosylates the death domains of proteins involved in death receptor signaling as TRADD, which block the signaling of these adaptors, thereby interrupting TNF-α-induced activation of NF-κB (Li, Zhang et al. 2013). NleH1 and NleH2 EPEC effectors interact with the ribosomal protein S3 (RPS3), which is a non-Rel subunit of NF-κB complexes. NleH1 inhibits nuclear translocation of RPS3, and blocks the NF-κB activation after TNF-α stimulation (Pham, Gao et al. 2012). NleC is a zinc metalloprotease that directly cleaves p65 subunit in its N-terminal domain (Yen, Ooka et al. 2010). NleC mediates proteolysis of p65 late in infection and results in proteasome-independent degradation of p65, suppress the subsequent NF-κB activation, and impair secretion of cytokines (Muhlen, Ruchaud-Sparagano et al. 2011). NleC degrades free p65 and also IκB-bound p65, although it has been proposed that free p65 is the primary target (Baruch, Gur-Arie et al. 2011, Santos and Finlay 2015). NleC also targets p50 and IκBα for proteasome-independent degradation (Muhlen, Ruchaud-Sparagano et al. 2011). NleC and NleD interfere with the MAPK proinflammatory pathway resulting in decrease cytokines secretion (Baruch, Gur-Arie et al. 2011, Sham, Shames et al. 2011). Other effectors block the host immune response through other mechanisms. NleA subdues host IL-1β secretion in a NF-κB-independent manner; NleA interaction with NLRP3 (Nod-like Receptor 3) disrupts the posterior inflammasome activation (Yen, Sugimoto et al. 2015). The product of the *lifA* gene, which encodes a toxin called lymphostatin, inhibits IL-2 and IL-4 production and lymphocyte proliferation and has also been reported to be injected by the T3SS (Klapproth, Scaletsky et al. 2000, Deng, Yu et al. 2012).

5.3.3.3 Regulation of cell survival and apoptosis

The epithelial cell death response to microbial infection is pivotal for pathogens and the host. Pathogens that are colonizing the epithelium need to prevent cell death to preserve their replicative foothold; by contrast, the host needs to eliminate infected cells in order to minimize tissue damage (Kim, Ashida et al. 2010). During infection of intestinal epithelial cells, surfaces properties of EPEC are recognized by cell surface death receptors and induce extrinsic apoptotic pathways, while T3SS effectors (Map and EspF) trigger intrinsic apoptotic pathways (Wong, Pearson et al. 2011, Santos and Finlay 2015) (Figure 7). EspF is targeted to the mitochondria by an N-terminal target sequence and destabilizes mitochondrial membrane potential leading to cytochrome *c* release, activation of caspases and downstream intrinsic apoptosis (Nougayrede and Donnenberg 2004, Nagai, Abe et al. 2005). Map also localizes in the mitochondria through a N-terminal mitochrondria targeting sequence, inducing mitochondria disruption, cytochrome *c* release and the subsequent induction of apoptosis (Kenny and Jepson 2000, Ma, Wickham et al. 2006). Interestingly, early stages of apoptosis can be observed during EPEC infection, but late-stages are not evident, and this is because EPEC translocate effector proteins that antagonize these pro-apoptotic effects (Crane, McNamara et al. 2001, Wong, Pearson et al. 2011). NleD and NleB interfere with the pro-apoptotic death receptor signaling and disrupt the downstream extrinsic apoptosis (Baruch, Gur-Arie et al. 2011, Pearson, Giogha et al. 2013). NleH1/NleH2 inhibit caspase-dependent intrinsic apoptosis in infected host cells, reducing levels of active caspase-3 and thereby preventing cell death. This ability appears to be largely due to the interaction of NleH1 and NleH2 with the anti-apoptotic protein, Bax inhibitor-1 (BL-1)(Hemrajani, Berger et al. 2010, Royan, Jones et al. 2010). EspZ also inhibits intrinsic apoptosis and promotes host cell survival. EspZ localizes in the mitochrondria and stabilizes mitochondrial membrane potential, counteracting cytotoxicity effects of Map and EspF, preventing the release of cytochrome *c* and the subsequent induction of intrinsic apoptosis (Roxas, Wilbur et al. 2012). EspZ also localizes to the cytoplasmic face of the plasma membrane at the site of bacterial attachment and interact with EspD translocator. It has been proposed that EspZ prevents cell death by downregulating protein translocation; consistently an EPEC Δ*espZ* mutant was highly cytotoxic (Berger, Crepin et al. 2012). NleF effector protein directly inhibits caspases involved in both intrinsic and extrinsic apoptosis pathways, including caspases-4, -8 and -9 (Blasche, Mortl et al. 2013). EPEC effectors are injected in a regulated manner to guarantee the infection, such as the prosurvival effector EspZ is translocated earlier, whereas the pro-apoptotic EspF and Map follows later, the translocation order indicates that

EspZ is able to act first preventing cell death (Mills, Baruch et al. 2008).

6. Models to study EPEC infection

Because EPEC is a human pathogen primarily affecting infants, experimental models are needed to study the infection process. The majority of the information about EPEC infection and induction of actin pedestal formation have been obtained from *in vitro* tissue culture cell models and most of these culture cell lines are nonpolarized cell lines and are not from intestinal origin (Knutton, Baldwin et al. 1989, Berger, Crepin et al. 2009, Berger, Crepin et al. 2012). Therefore, the physiological relevance of the finding discovered in culture cell lines needs confirmation in more biologically relevant models. Hence, some studies have been carried out in adults human volunteers but they used a large bacterial doses that represents an artificial situation (Donnenberg, Tacket et al. 1993, Nataro and Kaper 1998). Although EPEC does not infect most laboratory animal models, several studies have reported that mice were susceptible to infection by *E. coli* pathogens(Hecht, Marrero et al. 1999, Savkovic, Villanueva et al. 2005). Thus, a study indicates that mice infection by EPEC induced intestinal inflammation and some microvillus effacement but without intimate bacterial attachment with the mice intestinal surface (Savkovic, Villanueva et al. 2005). By contrary, other studies indicate that EPEC do not induce A/E lesion in the mouse intestine and that EPEC establishes a commensal relationship with the mouse host (Frankel, Phillips et al. 1996, Klapproth, Sasaki et al. 2005, Mundy, Girard et al. 2006). Hence, the use of adult mice infection model is questionable. The A/E lesion induced by EPEC has also been studied in ligated intestinal loops of pigs and rabbits and in gnotobiotic pigs and newborn mice, which lack a mature microbiome and immune system, but except the pig model none of these models fully reproduce the phenotype of A/E lesion observed in the human intestine (Moon, Whipp et al. 1983, Dupont, Sommer et al. 2016). Importantly, *ex vivo* infection of human cultured intestinal biopsies (IVOC of In Vitro Organ culture) with EPEC induces A/E lesions that are undistinguishable of those observed in intestinal biopsies of patients with EPEC diarrhea. Because of this, the IVOC assay has been proposed as a model to study EPEC infection in closer circumstances to natural EPEC infection (Knutton, Lloyd et al. 1987). Several studies have also revealed that conclusions made during infection of cultured cell lines *in vitro* cannot be completely extrapolated to infection of IVOC. For instance, some EPEC strains failed to induce fluorescence actin staining (FAS) on Hep-2 cells but produced typical A/E lesions on human IVOC (Knutton, Shaw et al. 2001). In addition, an EPEC strain expressing TirY474S, which disrupts the most critical phosphorylation residue for actin assembly in cell culture, displayed

classical A/E lesion during IVOC infections of human duodenal biopsies (Schuller, Chong et al. 2007). Hence, infection studies with human IVOC may be closer to the actual *in vivo* situation in human intestine.

7. EPEC treatment and vaccines

The primary goal to treatment of EPEC diarrhea is to prevent dehydration by correcting body fluid and electrolytes imbalance. Oral rehydration may be sufficient for milder cases, but more severe cases require parental rehydration (Nataro and Kaper 1998). Persistent infections may require the use of antibiotics. Antibiotics have been useful in many cases, but several clinical isolates exhibiting a high degree of resistance to standard antibiotics (Hill, Phillips et al. 1991, Subramanian, Selvakkumar et al. 2009, Croxen, Law et al. 2013). EPEC antibiotic-resistant strains have been found across many continents, with reported cases in Mexico (Gomez-Aldapa, Cerna-Cortes et al. 2016), Brazil (Scaletsky, Souza et al. 2010), United Kingdom (Jenkins, Smith et al. 2006), Singapore (Lim, Ngan et al. 1992) and the United States (Moyenuddin, Wachsmuth et al. 1989). EPEC is the etiological agent of important diarrhea, however there are not vaccines available to control its spread. In a study conducted with adult human volunteers, they demonstrated that previous infection with EPEC E2348/69 reduces the severity of subsequent illness upon reinfection with the homologous strain. No evidence of protective immunity was observed, but it was probably because of the high inoculum dose necessary to induce efficient colonization in adults (Donnenberg, Tacket et al. 1998). Antibodies from maternal colostrum and serum samples have been shown to recognize EPEC surface antigens such as BFP, Intimin, and to the secreted proteins EspB and EspA (Parissi-Crivelli, Parissi-Crivelli et al. 2000). Therefore these virulence proteins are good candidates for vaccine development against EPEC infection. Human volunteer studies have shown that 36% of individuals who ingested an EPECΔ*eae* strains still developed diarrhea (Donnenberg, Tacket et al. 1993). Other study with adult volunteers, 10% of individuals who ingest Δ*espB* mutant developed diarrhea compared to 90% of diarrhea in individuals who ingested the WT strain (Tacket, Sztein et al. 2000). In a study conducted with Δ*bfp* mutant, it was still able to colonize and cause diarrhea in some cases although it was less virulent than the WT strain (Bieber, Ramer et al. 1998). These data indicate that more than one antigenic factor need to be deleted in EPEC to prevent infection. An alternative strategy would be to develop an attenuated EPEC strain that fails to cause diarrhea, but that keeps adherence factors to attach to the epithelial intestine surface and all antigens to induce an effective protection against the infection.

OBJECTIVES

Pathogenic bacteria such as EPEC have evolved to acquire virulence factors to compete with the commensal flora and to overcome the host immune response. Among these skills the acquisition of the T3SS allows EPEC to translocate effector proteins into the host-cell and subvert cellular functions toward infection. Common research on EPEC effectors is conducted by generating loss of function mutant strains that are complemented with plasmids overexpressing these effectors, or by the ectopic expression in the host cell of individual effector proteins after plasmid transfection. Both situations are prone to effector overexpression, producing non-physiological levels of effectors inside the host cell, which could alter effector activities. In addition, effectors often have synergistic and overlapping functions that cannot be fully appreciated by single mutations and individual transfection experiments. Hence, it will be interesting for EPEC research the availability of mutant strains lacking all the effectors of the T3SS and that would enable the control reinsertion of specific genes encoding effector protein(s) in the chromosome, allowing the study of these effectors at physiological levels and without the background activity of other effector proteins. Lastly, generation of these effector mutant EPEC strains, which are expected to be highly attenuated in virulence, will be of interest for development of an EPEC vaccine and for the therapeutic delivery of specific proteins in human intestine.

Thus, the objectives of this thesis are:

1. To evaluate the applicability of the markerless gene deletion strategy for the appropriate genome manipulation of EPEC serotype O127:H6 strain E2348/69.

2. To generate an EPEC strain lacking of all known effector proteins of the T3SS, preserving the correct assembly and function of the T3SS injectisome.

3. To identify the minimum set of effectors that EPEC needs to induce actin-pedestal formation into HeLa cells.

4. To translocate individual effector proteins into HeLa cells from the effector mutant EPEC strains and to evaluate the phenotype elicited by these effectors.

5. To infect human intestinal biopsies with the effector mutant EPEC strains in order to identify the essential effectors needed to induce the A/E lesion formation in the mucosal tissue.

MATERIAL AND METHODS

1. Bacterial strains

The bacterial strains used in this work are listed in Table 1.

Table 1. Bacterial strains used in this work

Name	Genotype and relevant properties	Reference
DH10B-T1^{R}	(F- λ-) mcrA Δmrr-hsdRMS-mcrBC φ80lacZDM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK rpsL (StrR) nupG tonA	Thermo Fisher (Durfee, Nelson et al. 2008)
BW25141	*(F- λ-) Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), Δ(phoB-phoR)580, galU95, ΔuidA3::pir, recA1, endA9(del-ins)::FRT, rph-1, Δ(rhaD-rhaB)568, hsdR51.*	(Datsenko and Wanner 2000)
CC118 λ*pir*	Δ(*ara-leu*) *araD ΔlacX74 galE galK phoA20 thi-rpsE rpoB argE(Am) recA1, λpir*	(Herrero, de Lorenzo et al. 1990)
EPEC	EPEC 0127:H6 E2348/69	(Iguchi, Thomson et al. 2009)
EPECΔ*escN*	E2348/69 Δ*escN*	This work
EPECΔ*map*	E2348/69 Δ*map*	This work
EPECΔ*map. espG*	E2348/69 Δ*map* Δ*espG*	This work
EPECΔ*map. espGF*	E2348/69 Δ*map* Δ*espG* Δ*espF*	This work
EPEC9	E2348/69 Δ*map* Δ*espG* Δ*espF* Δ*espH*	This work
EPEC8	EPEC9 ΔIE5	This work
EPEC7	EPEC8 ΔIE6	This work
EPEC6	EPEC7 ΔIE2	This work
EPEC5	EPEC6 ΔPP2	This work
EPEC4	EPEC5 ΔPP3	This work
EPEC3	EPEC4 ΔPP4	This work
EPEC2	EPEC3 ΔPP6	This work
EPEC1	EPEC2 Δ*espZ*	This work
EPEC0	EPEC1 Δ*tir*	This work
EPEC2-LEE+	EPEC2+(*map*+*espH*+*espF*+*espG*)	This work
EPEC7Δ*lifA*-like	EPEC7 Δ*lifA*-like	This work
EPEC7Δ*nleE*	EPEC7 Δ*nleE*	This work
EPEC2+*map*	EPEC2+*map*	This work

EPEC1+*map*	EPEC1+*map*	This work
EPEC0+*map*	EPEC0+*map*	This work
EPEC2+*nleC*	EPEC2+*nleC*	This work
EPEC1+*nleC*	EPEC1+*nleC*	This work
EPEC0+*nleC*	EPEC0+*nleC*	This work
EPEC2+*espH*	EPEC2+*espH*	This work
EPEC1+*espH*	EPEC1+*espH*	This work
EPEC0+*espH*	EPEC0+*espH*	This work
EPEC2Δ*eae*	EPEC2 Δ*eae*	This work
EPEC1Δ*eae*	EPEC1 Δ*eae*	This work
EPECΔ*grlR*	E2348/69 Δ*grlR*::Amp^{R}.FRT	This work

2. Conditions for bacterial growth

Bacteria were grown at 37°C in Luria Bertani (LB) agar plates (1.5% w/v) (Bertani 1951), in liquid LB medium or in Dulbecco's Modified Eagle's medium (DMEM). When needed for plasmid or strain selection, antibiotics were added at the following concentrations: ampicillin (Amp) 150 µg/ml, except for strains expressing the Amp resistance gene from their chromosome, which were selected with Amp at 75 μg/ml; chloramphenicol (Cm) 30 µg/ml; kanamycin (Km) 50 µg/ml; tetracycline (Tc) 10 µg/ml; spectinomycin (Sp) 50 µg/ml.

2.1 Induction of the T3SS in EPEC

EPEC strains were grown overnight (o/n) static at 37°C in a flask with 5 ml of liquid LB, unless other wise indicated. Next day, cultures were diluted to a 0.05 OD_{600} in 5 ml of LB/DMEM in a well capped Falcon tubes (BD Biosciences) and incubated in agitation (180 rpm) for 4 h for the expression of the T3SS, these cultures were used for the analysis of the T3-secreted proteins to the supernatant. For the infection of cell cultures with EPEC strains, the o/n LB bacteria cultures were diluted 1:50 in 5 ml of DMEM serum free in 50 ml Falcon tubes and were grown at 37 °C and 5% CO_2, with the lid open in static during 2.5 h, unless otherwise indicated. EPEC strains harbouring *map, tir and nleC* for the analysis of individual translocation of Map, Tir and NleC were grown o/n with agitation at 37°C in a 30 ml tube SterilinTM with 5 ml of liquid LB. The o/n LB bacteria cultures were diluted 1:100 in 5ml of DMEM serum free in 30 ml tube SterilinTM and were grown at 37 °C and 5% CO_2, with the lid open and in static during 3 h.

Alternatively EPEC strains harbouring Tc^R plasmids used for the β-lactamase assay, were grown o/n at 37 ºC with agitation at 180 rpm with Tc and next day were diluted to 0.05 OD_{600} in 4 ml of DEMEM without Tc and grown in 6-well tissue culture plates (Falcon) during 3 h, at 37 ºC and 5% CO_2.

3. DNA constructs

E. coli DH10B-$T1^R$ strain was used as host for the cloning and propagation of plasmids with the pBR or pSC101-ts origins of replication. In the case of suicide pGE derivatives, harbouring the R6K-ori, the *E. coli* strains BW25141 or CC118-λpir were used, these strains express the π protein needed for plasmid replication (Stalker, Kolter et al. 1982).

Plasmids employed in this study are summarized in Table 2. Standard methods of DNA manipulation were used (Ausubel F.M. 2002). When indicated, DNA was synthesized by GeneArt (Life Technologies). All DNA constructs were verified by DNA sequencing (Secugen or StabVida). Oligonucleotides used in this work were obtained from Sigma and are described in Table 3. PCRs were performed with the Taq DNA polymerase (Roche, NZyTech) for standard amplifications in screenings and with the proofreading DNA polymerase Herculase II Fusion (Agilent Technologies) and Vent DNA polymerase (NEB) for cloning purposes. Details of plasmid constructions are described below.

Table 2. Plasmids used in this work

Plasmid	Relevant genotype and features	Reference	Primers
pCX340	(Tc^R) pBR ori, p*trc* promoter, for fusions to the β-lactamase	(Charpentier and Oswald 2004)	
$pEspF_{1-20}Bla$	pCX340 derivative; EspF secretion signal, residues 1-20 fused to the β-lactamase	(Blanco-Toribio, Muylder-mans et al. 2010)	
pACBSR	(Cm^R) p15A ori, pBAD promoter, I-*Sce*I endonuclease and λ Red genes	(Herring, Glasner et al. 2003)	

pACBSR-Sp	(Sp^R) p15A ori, pBAD promoter, I-*Sce*I endonuclease and λ Red genes	This work	
pGETS	(Km^R); pSC101-ts ori, polilynker flanked two I-*Sce*I restriction sites	This work	102, 103
pGE	(Km^R) R6K-ori, polylinker flanked by two I-*Sce*I restriction sites	(Pinero-Lambea, Bodelon et al. 2014)	
pGEΔ*map*	pGE derivative; with homology regions flanking the *map* gene of EPEC	This work	GeneArt, Life Technologies XhoI-SphI
pGEΔ*espG*	pGE derivative; with homology regions flanking the *espG* gene of EPEC	This work	3, 4, 5, 6
pGEΔ*espF*	pGE derivative; with homology regions flanking the *espF* gene of EPEC	This work	GeneArt, Life Technologies XhoI-SphI
pGEΔ*espH*	pGE derivative; with homology regions flanking the *espH* gene of EPEC	This work	11, 12, 13, 14
pGEΔIE5	pGE derivative; with homology regions flanking cluster of effector genes in IE5 (*espC* and *espG*) of EPEC	This work	15, 16, 17, 18
pGEΔIE6	pGE derivative; with homology regions flanking cluster of effector genes in IE6 (*espL*, *nleB*, *nleE*, e*fa1/lifA*) of EPEC	This work	21, 22, 23, 24

pGEΔIE2	pGE derivative; with homology regions flanking cluster of effector genes in IE2 (*efa1/lifA, nleE, nleB*, espL**) of EPEC	This work	27, 28, 29, 30
pGEΔPP2-Amp.FRT	pGE derivative; with Amp^R (flanked by FRTs) between the homology regions flanking cluster of effector genes in PP2 (*nleH, Cif*, espJ*) of EPEC	This work	33, 34, 35, 36, 57, 59
pGEΔPP3-Amp.FRT	pGE derivative; with Amp^R (flanked by FRTs) between the homology regions flanking *nleJ* in PP3 of EPEC	This work	39, 40, 41, 42, 57, 59
pGEΔPP4-Amp.FRT	pGE derivative; with Amp^R (flanked by FRTs) between the homology regions flanking cluster of effector genes in PP4 (*nleG, nleB, nleC, nleH*, nleD*) of EPEC	This work	45, 46, 47, 48, 57, 59
pGETSΔPP6-Amp.FRT	pGETS derivative; with Amp^R (flanked by FRTs) between the homology regions flanking cluster of effector genes in PP6 (*nleA/espL, nleH, nleF, espO*) of EPEC	This work	51, 52, 53, 54, 58, 59
pGEΔPP2	pGE derivative; with the homology regions flanking cluster of effector genes in PP2 (*nleH, Cif*, espJ*) of EPEC	This work	33, 34, 35, 36
pGEΔPP3	pGE derivative; with the homology regions flanking *nleJ* in PP3 of EPEC	This work	39, 40, 41, 42
pGEΔPP4	pGE derivative; with the homology regions flanking cluster of effector genes in PP4 (*nleG, nleB, nleC, nleH*, nleD*) of EPEC	This work	45, 46, 47, 48
pGEΔPP6	pGE derivative; with the homology regions flanking cluster of effector genes in PP6 (*nleA/espL, nleH ,nleF, espO*) of EPEC	This work	51, 52, 53, 54

pGEΔ*espZ-1*	pGE derivative; with the homology regions flanking the *espZ* gene of EPEC	This work	71, 74, 110, 111
pGEΔ*espZ-2)*	pGE derivative; with the homology regions flanking the *espZ* gene and RBS	This work	71, 72, 73, 74
pGEΔ*tir*	pGE derivative; with the homology regions flanking the *tir* gene of EPEC	This work	77, 78, 79, 80
pGE*minCD*-Amp.FRT	pGE derivative; with the homology regions flanking the *minCD* genes of *E. coli* K-12 and the *bla* gene (Amp^R) flanked by two FRT sites inserted between homology regions	Carlos Piñero, Thesis 2014	
pGEΔ*escN*	pGE derivative; with the homology regions flanking the *escN* gene of EPEC	This work	60, 61, 62, 63
pGE+*map*	pGEΔ*map* derivate; with *map* plus HR as in the chromosome	This work	66, 67
pGE+*espH*	pGEΔ*espH* derivate; with *espH* plus HR as in the chromosome	This work	11, 68
pGE+*espF*	pGEΔ*espF* derivate; with *espF* plus HR as in the chromosome	This work	69, 70
pGE+*espG*	pGEΔ*espG* derivate; with *espG* plus HR as in the chromosome	This work	3, 6

pGEΔPP4+*nleC*	pGEΔPP4 derivate; with the intergenic region between *nleG* and *nleB* fused to *nleC* plus HR as in the chromosome	This work	83, 84, 85, 86
pGEΔ*lifA*-like	pGEΔIE2 derivative; with homology regions flanking *lifA-like* gene in IE2 of EPEC	This work	87, 88
pGEΔ*nleE*	pGE derivative; with the homology regions flanking the *nleE* gene in IE2 of EPEC	This work	90, 91, 92, 93
pGEΔ*eae*	pGE derivative; with the homology regions flanking the *eae* gene of EPEC	This work	96, 97, 98, 99
pGETSΔ*grlR*-Amp.FRT	pGETS derivative; with Amp^R (flanked by FRTs) between the homology regions flanking *grlR* regulator gen of EPEC	This work	104, 105, 106, 107
pKD46	(Amp^R) pSC101-ts ori, pBAD, Red recombinase expression plasmid.	(Datsenko and Wanner 2000)	
pGE-*espB*Δmid	pGE derivative; plus EPEC *espB* with deletion of amino acid 159-218.	This work	112, 113, 114, 115

Table 3. Oligonucleotides used in this work

Number	Name	Sequence (5´-3´)
1	PF.CesF82	cgtgaaaagcgagggcgtcagtttg
2	PR-tir.86	gcgccgtctgtttgtgaaggtagtg
3	F.XhoI.espG. HR1(2)	ccggttCTCGAGgcctctggaatagttgcttgctttacactcag
4	R.espG.HR1.Fus	tatgatgctataataaaactttattaatcaaaaccaata-atagaaatc
5	F.espG.HR2.Fus	taaagttttattatagcatcatatagtgtcaataatatacaa-gatatttatagcgg
6	R.sphI.espG. HR2(2)	cgacatGCATGCgaacaagggacaaatagctgaacaagta-accgcg
7	F.check.ΔespG	cgattcatcggacagaatcatcagactttcat
8	R.check.ΔespG	tcggcgaaagaggatctgccatacatcaag
9	PF.escF.179	cgataaaagatctggtctcaaccatttctaacc
10	PR.IE.espF	gtaatacggaaatacattgag
11	F.XhoI.espH. HR1(3)	cggCTCGAGccggaaggtgatgtgtcagttgatgatg
12	PR.EspH.HR1.Fus	cataaaataatactcctgattaatcacatacta
13	PF.EspH.HR2.Fus	tgattaatcaggagtattattttatgctgttttctttttctcc
14	R.sphI.espH.HR2	gcccGCATGCctgacgccctcgcttttcacgataacg
15	F.XhoI.*espG2*.HR1	cccgCTCGAGtgaaggcgaaaaagatgattg
16	R.SacI.*espG2*.HR1. Fus	cagatttaaacgtctgGAGCTCtacacatccttttattc
17	F.SacI.*espC*.HR2. Fus	gaataaaaaggatgtgtaGAGCTCcagacgtttaaatctggc
18	R.speI.*espC*.HR2	ccggACTAGTcagtagctgaagtaatggtattgc
19	F.check.ΔIE5	cttaaccagataagagttaaatg
20	R.check.ΔIE5	gccacgggtaaaaagtggcgttttcg
21	F.XhoI.*espL*.HR1	ccggCTCGAGgtgaagccaccctcctctcccctggcg
22	R.SacI.*espL*.HR1	cagagattttaaGAGCTCaacaacatttgtgcctgaggaaag
23	F.SacI.*lifA*.HR2	cacaaatgttgttGAGCTcttaaaatctctgttaaagatg
24	R.SpeI.*lifA*.HR2	cctagACTAGTgtcagattctgaccagacg
25	F.check.ΔIE6	gtcggtttttccgtcccaccgggatatc
26	R.check.ΔIE6	gttaccaattcttgtctaatggc
27	F.XhoI.*lifA*-like. HR1	ccggCTCGAGggtgtgcaggatacctgcctctatcatc
28	R.SacI.*lifA*-like.HR1	cataccatctttatgGAGCTCtagttttgcacaatatattc
29	F.SacI.*espL**.HR2	gtgcaaaactaGAGCTCcataaagatggtatgacc
30	R.SpeI.*espL**.HR2	cctagACTAGTtcatgattgattagctaaccagg

31	F.check.ΔIE2	cagccagaaaaatgtggtgtttaac
32	R.check.ΔIE2	ctgtttacctctttctcagggagtttag
33	F.XhoI.PP2.HR1.n	cccCTCGAGagtgtgtggtaagaccgctg
34	R.PP2.HR1.n	gaacgtgaaacgctGAGCTCccccattatccgagctag
35	F.PP2.HR2.n	cggataatgggggagCTCAGCgtttcacgttcagc
36	R.sphI.PP2.HR2	cgcgGCATGCccgcctcctttcctccccggtctg
37	F.check.ΔPP2	ctgaagtccggctggagtgagtg
38	R.check.ΔPP2.n	gggtaatcacgcaggtggtgatac
39	F.XhoI.nleJ.HR1	cccgCTCGAGgttgtaagtaccccgcttaggtg
40	R.SacI.nleJ.HR1.n	ctactcctcataGAGCTCTtacgggtaaaagcattctttattc
41	F.SacI.nleJ.HR2.n	gcttttacccgtaaGAGCTCtatgaggagtagcaaagtg-cactc
42	R.SphI.nleJ.HR2	cgcgGCATGCcatgcaccacctttatccag
43	F.check.ΔnleJ	gagacataaattcctaactgtg
44	R.check.ΔnleJ	ggtgaggtacaaccgcaaacac
45	F.XhoI.PP4.HR1.n	gcgCTCGAGccgctgcaaatcctgcgtgc
46	R.SacI.PP4.HR1.n	caagaaacacagGAGCTCatatgtgatactaaccg
47	F.SacI.PP4.HR2.n	gtatcacatatGAGCTCctgtgtttcttgtgc
48	R.SphI.PP4.HR2.n	cgcgGCATGCccggcagacttgctacctgc
49	F.check.ΔPP4.n	cattctggagtcagatgagaatgg
50	R.check.ΔPP4	cagatatcgccctggttgatag
51	F.XhoI.PP6.HR1	cccgCTCGAGatttgtggatgacatttgttgtggacc
52	R.SacI.PP6.HR1.n	cacactagatcGAGCTCgttggacaacggcatccaaatc
53	R.SacI.PP6.HR2.n	ccgttgtccaacGAGCTCgatctagtgtgattacaatc
54	R.SphI.PP6.HR2.n	cgcGCATGCccactttaactgcatgacagg
55	F.check.ΔPP6	gccgatacagtgcgtggtgaggc
56	R.check.ΔPP6.n	ggtttacattgttctaccacaatag
57	F.SacI.HindIII. Amp-FRT	cgcGAGCTCAAGCTTgaagttcctatactttctagaga-ataggaacttcggaataggaacttcatgagtaaacttggtct-gac
58	F.SacI.NdeI.Amp-FRT	cgcGAGCTCCATATGgaagttcctatactttctagaga-ataggaacttcggaataggaacttcatgagtaaacttggtct-gac
59	R.SacI.SpeI.Amp-FRT	cgcGAGCTCACTAGTgaagttcctattcc-gaagttcctattctctagaaagtataggaacttctcggggaaat-gtgcgcgg
60	F.XhoI.*escN*.HR1	ggccCTCGAGtgtgaaagagctgcagcgccagc
61	R.SacI.*escN*.HR1	gggcGAGCTCttaccgttcctaatactttaag
62	F.SacI.*escN*.HR2	gggcGAGCTCgtatgttggacagaattttatctattcg

63	R.SphI.*escN*.HR2	gacatGCATGCgattccgttactacatttaattg
64	F.check.Δ*escN*	cggcgtacaagaaacgcgttatttg
65	R.check.Δ*escN*	cgaaaacatagtctttttttatg
66	F.XhoI.*map*.HR1	gcgCTCGAGagatctttgcaaaattgttcattc
67	R.SphI.*map*.HR2	gccGCATGCacacgttcctttatattactatg
68	R.SpeI.espH.HR2	gcgACTAGTctgacgccctcgcttttcacgataacg
69	F.XhoI.*espF*.HR1.	ggccCTCGAGtaagcttcccaaatatgcgg
70	R.SphI.*espF*.HR2	cgccGCATGCcttcaggaaaaatataaccagataac
71	F.XhoI.*espZ*.HR1	ccggttCTCGAGacagtgcttgcatctgattagcttctttttctg
72	R.SacI.*espZ*.HR1.new.E	gcgcGAGCTCcataaaaatagagaggtaatggatgcattatgc
73	F.SacI.*espZ*.HR2.new.E	cgcgcGAGCTCctaatttagacatttacctgg
74	R.SphI.*espZ*.HR2	cgacatGCATGCgcgacaggcgcatcaacgtcgtaatcaac
75	F.check.Δ*espZ*	cctctttttccacactgagtgtcatatttcc
76	R.check.Δ*espZ*	gttaccgaaggagtaaataatgtcacccgc
77	F.XhoI.*tir*.HR1.new	cgcgCTCGAGggggaaacttactgcgctgttatttttttc
78	R.SacI.*tir*.HR1.new	gcgcGAGCTCacatatatccttttatttagaaatttg
79	F.SacI.*tir*.HR2.Fus	aaggatatatgtGAGCTCatatatctgtgagtatttag
80	R.SphI.*tir*.HR2	cgcgGCATGCgtttgggctccaccacaatgag
81	F.check.Δtir	ctacccagctactacaggccgtagc
82	R.check.Δtir	caatcctaaaccagcactaagc
83	F.SacI.intergenic.nlGB	GCGCgagctcaataaatattacccaagc
84	R.Fus.intergenic.nlGG	ctatatcaaattcattcgtcgtcctgtttatcc
85	F.Fus.*nleC*	gataaacaggacgacgaatgaatttgatatagtttatttttg
86	R.SacI.*nleC*	gcgcGAGCTCaaaatgtatgaatagtaac
87	F.SacI.*lifA*-like.HR2	ggccGAGCTCctgcaaaggtttagatattaac
88	R.SpeI.lifA-like.HR2	ggccACTAGTagaagctcagcaacttgtgtaagg
89	R.check.Δ*lifA*-like	gtgaaatgggagaaatctttagctac
90	F.XhoI.*nleE*.HR1	ggccCTCGAGctgcaaaggtttagatattaac
91	R.SacI.*nleE*.HR1	cagttcatggtaaGAGCTCagaagctcagcaacttgtg
92	F.SacI.HR2.*nleE*	gctgagcttctGAGCTcttaccatgaactgc
93	R.SpeI.*nleE*.HR2	ggccACTAGTccctgccagtgagaggg
94	F.check.Δ*nleE*	catattccggatgttctttgatac
95	R.check.Δ*nleE*	gagcagatgtggatttcagcatg
96	F.XhoI.*eae*.HR1	ccggCTCGAGcgttatctgatgccaatgacg

97	R.SacI.*eae*.HR1	gatatttattaaatGAGCTCgtttgggctccaccacaatgag
98	F.SacI.*eae*.HR2	gtggagcccaaacGAGCTCatttaataaatatctaatcattg
99	R.SpeI.eae.HR2	ggccACTAGTagatccttgccattataaatgc
100	F.check.Δ*eae*	gcggaaaaaattggtgttg
101	R.check.Δ*eae*	caatgaactggcatcag
102	F.AscI.repA101	cttGGCGCGCCgttagtcttgatgcttcactg
103	R.I-*Sce*I.XhoI. oriR101	cttGGCGCGCCgttagtcttgatgcttcactg
104	F.XhoI.HR1.*grl*R	gcggCTCGAGttctactgcatttggtgtattaaag
105	R.SacI.HR1.*grl*R	ggccGAGCTCactaacctcactcctttcaatttg
106	F.SacI.RBS.HR2. *grl*R	ggccGAGCTCaactttaagaaggagatatatccatggaatc-taaaaataaaaatggcg
107	R.SphI.HR2.*grlR*	ccggGCATGCctaactctccttttccgcctc
108	F.check.Δ*grlR*.n.2	gttagaggctaagatataac
109	R.check.Δ*grlR*.n.2	gtgaagtgctcccaaagttg
110	R.HR1.*espZ*.Fus.	ggagaaagatcatcatcgcatcgcataaaaatagagaggta-atggatgc
111	F.HR2.*espZ*.Fus	gcgatgcgatgatgatctttctccttttgtctaatttagacatt-tacctgg
112	F.XhoI.HR1.espB. mid	cccCTCGAGcagctgatgtctgattctgcg
113	R.HR1.*espB*.Δmid	gcagtaaagcgactttcggaagccttcgccag
114	F.HR2.*espB*.Δmid	cgaaggcttccgaaagtcgctttactgctgctgcc
115	R.sphI.HR2. *espB*.Δmid	cgcGCATGCctccttctgtattgtgtacc
116	qPCR-lifAhomo Rev	acgccgtgataaaatactccg
117	qPCR-lifAhomo For	cgatacaacgcccttcattg
118	qPCR tir For	cggaatagtctatcggctcatc
119	qPCR tir Rev	tactttggataccttgccctg
120	qPCR nleE2 For	atggttgttgtgtacagaaatgac
121	qPCR nleE2 Rev	ctgcctttaaatctggtaactcataat

3.1 Plasmid constructions

3.1.1 Thermo sensitive plasmid for genome engineering (pGETS)

The pGETS is a Km^R plasmid of 2817 bp with a thermo-sensitive pSC101-ori; it contains two I-*Sce*I sites flanking a multiple cloning site (MCS) with the following restriction sites: *Xho*I, *Bsa*I, *Sac*I, *Nde*I, *Xba*I, *Hind*III, *Spe*I, *Avr*II and *Sph*I. This plasmid was constructed ligating two DNA fragments: one encompassing the Km resistance cassette and the MCS (digested from the pGE) and the other with the *repA101* and the pSC101-ori. The later fragment was amplified from the pKD46 thermo-sensitive plasmid.

3.1.2 Design of pGE and pGETS derivate plasmids for EPEC genome engineering

Derivative plasmids of pGE and pGETS harbor integrative cassettes for the deletion of genes sequences in the bacterial chromosome, and a backbone with a R6K or pSC101 origin of replication respectively. Two homology regions (HRs) flanking the gene to be deleted and cloned between *Xho*I and *Sph*I or *Spe*I endonucleases compose the integrative cassette. When indicated, the constructions harbour a *Sac*I site between the HRs. When positive selection of the deletion was needed, an Amp^R cassette flanked by Flippase Recognition Target (FRT) sequences was cloned in the *Sac*I site between the HRs, the Amp.FRT cassette was amplified from the pGE*minCD*-Amp.FRT (Piñero-Lambea C., PhD Thesis). The HRs were designed for the deletion of effector genes from the start codon to the stop codon (Figure 8). In the case of deletion of *espZ* the plasmid pGEΔ*espZ* was designed to delete *espZ* and its ribosome-binding site (RBS), leaving the next gene of the LEE2 operon (*escI*) with its own RBS.

To insert genes in the bacterial chromosome, pGE plasmids were constructed with a DNA fragment encompassing the corresponding wild type *orf* of the effector and the flanking HRs. These cassettes for the insertion of *espH*, *map*, *espF* and *espG* were amplified from the chromosome with the external oligonucleotides used to amplified the HRs in the case of gene deletion. For the insertion of *nleC* a fusion PCR was done between the *nleG* and *nleB* intergenic region and the *nleC* gene. This fusion was cloned in the *S*acI site between the HRs of the respective pGE. To see details of the pGE plasmids for insertion see Table 2 and Table 3.

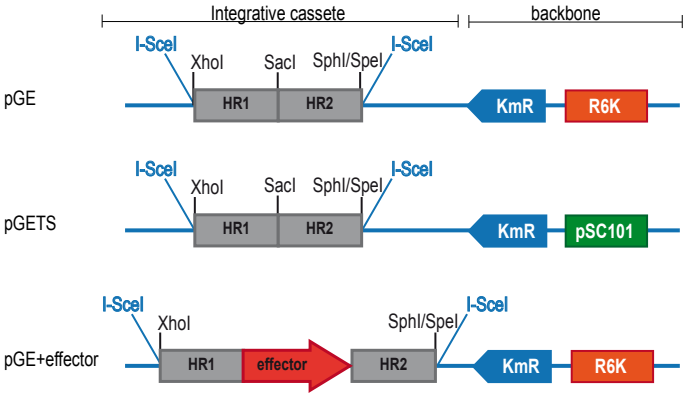

Figure 8. Plasmid for gene(s) deletion and gene integration linear maps are displayed. pGE and pGETS plasmid vectors for E.coli genome engineering, with an integrative cassette with the mutant allele. pGE contains a suicide origin of replication R6K (pir dependent). And pGETS contains a thermo-sensitive origin of replication pSC101. Plasmids pGE+effector carry the corresponding effector gene with HRs for gene integration.

4. Genome engineering of EPEC strains

EPEC mutant strains (Table 1) were generated using a markerless gene deletion or integration strategy, which is based in homologous recombination of suicide plasmids with I-*SceI* restriction sites and homology regions to the targeted gene, followed by the expression *in vivo* of the I-*SceI* endonuclease (Posfai, Kolisnychenko et al. 1999). Genome modifications, suicide plasmids and oligonucleotides used to screen the mutant strains are summarized in Table 4.

Table 4. Modifications of the genome using pGE and pGETS plasmids

Strain to be modified	Plasmid to be integrated	Resulting Strain	Checking primers
EPEC	pGEΔ*escN*	EPECΔ*escN*	64, 65
EPEC	pGEΔ*map*	EPECΔ*map*	1, 2
EPECΔ*map*	pGEΔ*espG*	EPECΔ*map.espG*	7, 8
EPECΔ*map.espG*	pGEΔ*espF*	EPECΔ*map.espGF*	9, 10
EPECΔ*map.espGF*	pGEΔ*espH*	EPEC9	11, 14
EPEC9	pGEΔIE5	EPEC8	19, 20
EPEC8	pGEΔIE6	EPEC7	25, 26
EPEC7	pGEΔIE2	EPEC6	31, 32
EPEC6	PGEΔPP2-Amp. FRT	EPEC5-Amp.FRT	37, 38

EPEC5-Amp.FRT	PGEΔPP2	EPEC5	37, 38
EPEC5	pGEΔPP3-Amp. FRT	EPEC4-Amp.FRT	43, 44
EPEC4-Amp.FRT	pGEΔPP3	EPEC4	43, 44
EPEC4	pGEΔPP4-Amp. FRT	EPEC3-Amp.FRT	49, 50
EPEC3-Amp.FRT	pGEΔPP4	EPEC3	49, 50
EPEC3	pGETSΔPP6-Amp.FRT	EPEC2-Amp.FRT	55, 56
EPEC2-Amp.FRT	pGEΔPP6	EPEC2	55, 56
EPEC2	pGEΔespZ	EPEC1	75, 76
EPEC1	pGEΔtir	EPEC0	
EPEC7	pGEΔ*lifA*-like	EPEC7Δ*lifA*-like	31, 89
EPEC7	pGEΔ*nleE*	EPEC7Δ*nleE*	94, 95
EPEC2	pGE+*map*	EPEC2+*map*	1, 2
EPEC1	pGE+*map*	EPEC1+*map*	1, 2
EPEC0	pGE+*map*	EPEC0+*map*	1, 82
EPEC2	pGE+*nleC*	EPEC2+*nleC*	49, 50
EPEC1	pGE+*nleC*	EPEC1+*nleC*	49,50
EPEC0	pGE+*nleC*	EPEC0+*nleC*	49, 50
EPEC2	pGE+*espH*	EPEC2+*espH*	11, 14
EPEC1	pGE+*espH*	EPEC1+*espH*	11, 14
EPEC0	pGE+*espH*	EPEC0+*espH*	11, 14
EPEC2+*map*	pGE+*espH*	EPEC2+*map*+*espH*	11, 14
EPEC2+*map*+*espH*	pGE+*espF*	EPEC2+*map*+*espH*+*espF*	9, 10
EPEC2+*map*+*espH*+*espF*	pGE+*espG*	EPEC2-LEE+	7, 8
EPEC2	pGEΔ*eae*	EPEC2Δ*eae*	100, 101
EPEC1	pGEΔ*eae*	EPEC1Δ*eae*	100, 101
EPEC	pGEΔ*grlR*	EPECΔ*grlR*	108, 109
EPEC	pGEΔ*espZ*(1)	EPECΔ*espZ*(1)	75, 76
EPEC	pGEΔ*espZ*(2)	EPECΔ*espZ*(2)	75, 76
EPEC2	pGE-*espB*Δmid	EPEC2Δmid	112, 115
EPEC1	pGE-*espB*Δmid	EPEC1Δmid	112, 115

4.1 Use of suicide pGE plasmids with R6K origin of replication

The EPEC strain to be modified was initially transformed with a plasmid pACBSR (Sp^R or Cm^R) (Herring, Glasner et al. 2003), expressing the I-*Sce*I and λ Red

proteins under the control of the PBAD promoter (inducible with L-arabinose), and subsequently electroporated with the corresponding pGE-based suicide vector (Km^R). Colonies that grew in LB-Km-Cm (or Sp) plates had integrated the suicide plasmid in their genome (cointegrants) by recombination. Correct integration was verified by PCR. The colonies were grown for 6 h in LB-Cm (or Sp) liquid medium containing L-arabinose 0.4% (w/v) with agitation (180 rpm) to induce the expression of I-*Sce*I and to cause a double-strand break in the genome that promotes a second step of recombination. A sample of these cultures was streaked on LB-Cm (or Sp) agar plates and incubated o/n to isolate individual colonies, which were replicated in LB-Cm/Sp and LB-Km-Cm/Sp agar plates to identify Km-sensitive colonies. The resolution of the cointegrates can either revert the sequence to the WT or maintain the mutant allele sequence, depending on the site of the crossover. Using specific primers (Table 4), the mutants harboring deletion or insertion of genes were identified by PCR.

Deletion of the cluster of genes present in EPEC prophages (PPs) required a positive selection to select the mutant strains. In these cases, pGE-Amp.FRT vectors were initially used to carry out the deletion of cluster of genes present in PP2, PP3 and PP4, followed by a second mutation step with the corresponding pGE-deletion vector to remove the Amp.FRT cassette. After electroporation of EPEC strains having pACBR plasmid with the corresponding pGE-Amp.FRT, the cointegrants were plated in LB-Amp-Km-Cm (or Sp). Individual colonies were grown for 6 h in LB-Amp-Cm (or Sp) liquid medium containing L-arabinose 0.4% (w/v) with agitation (180 rpm), to induce the double-strand brake and the second recombination. A sample of these cultures was streaked on LB-Amp-Cm (or Sp) agar plates and incubated o/n to isolate individual colonies, which were replicated in LB-Amp-Cm (or Sp) and LB-Amp-Km-Cm (or Sp) agar plates to identify Amp^R and Km^S colonies. Deletions of genes were identified by PCR (Table 4). The Amp-FRT resistance cassette was later removed as explained in section 4.3.

4.2 Use of suicide pGETS plasmids with thermo-sensitive pSC101 origin of replication

EPEC strains harbouring pACBSR-Cm^R were transformed with the pGETS-Km^R vector designed for gene deletion, which contains the thermosensitive origin of replication pSC101. The transformants were plated in LB-Km-Cm and incubated o/n at 30°C to obtain individual colonies. Several colonies were restreaked in LB-Km-Cm plates and incubated at 42°C during 7-9 h (to induce plasmid integration), and the plates were kept for additional 12-24 h at 37°C. Between the small and large colonies grown on the plates, the larger colonies were selected and restreaked in

LB-Km-Cm plates and incubated o/n at 37°C. The colonies were grown for 6 h in LB-Cm liquid medium containing L-arabinose 0.4% (w/v) with agitation (180 rpm) to induce the expression of I-*Sce*I and to cause a double-strand break in the genome to promote a second step of recombination. A sample of these cultures was streaked on LB-Cm agar plates and incubated o/n to isolate individual colonies, which were replicated in LB-Cm and LB-Km-Cm agar plates to identify Km^S colonies. Using specific primers (Table 4), the mutants harboring deletion or insertion of genes were identified by PCR screening.

The pGETS can also be integrated by a single step of recombination; EPEC3 mutant harbouring pACBSR-Sp^R was transformed with pGETSΔPP6-Amp.FRT and the transformants were plated and incubated o/n at 30 °C. Individual colonies were grown at 30 °C in liquid LB medium with agitation (180 rpm) until the cultures reached an OD_{600} of 0.5. Then, L-arabinose 0.4% (w/v) was added to induce the expression of I-*Sce*I and the λ Red proteins, which triggered the excision from the vector on the integrative cassette as lineal DNA, protect it from digestion and assisted in the posterior double recombination event. The temperature was shifted to 37 °C to avoid replication of pGETS plasmid. The cultures were further grown during 4 h and streaked LB-Amp-Sp to select the mutant strains that had deleted the cluster of genes present in PP6 and also to maintain the selection of the pACBSR plasmid. The grown colonies were analyzed by PCR with specific oligonucleotides to check the correct deletion, Amp.FRT resistance cassette was later removed.

4.3 Removal of the Amp.FRT cassette

After deletion of the clusters of effector genes localized in EPEC PPs using positive selection with Amp.FRT cassette, we deleted the Amp.FRT cassette of the resulting strains. Expression of flippase recombinase from the pCP20 is the conventionally method to remove the Amp.FRT cassette from bacterial chromosomes but leaves the FRT site as a "scar" in the chromosome. In addition, transformation of pCP20 is not stable in EPEC for unknown reasons. Hence, excision of the Amp.FRT strains was done using the homologous recombination strategy that leaves no FRT scars in the chromosome. The EPEC mutants with the Amp.FRT cassette and pACBSR-Sp^R (or Cm^R) were transformed with their respective pGE deletion plasmid lacking the Amp.FRT cassette. The transformants were plate in LB Amp-Km-Sp (or Cm) and were incubated at 37 °C o/n. Colonies that grew in LB-Amp-Km-Sp (or Cm) are the cointegrants and had two copies of the mutant allele with and without the Amp.FRT cassette respectively. Correct integration was verified by PCR. The colonies were grown for 6 h in LB-Cm (or Sp) liquid medium containing

L-arabinose 0.4% (w/v) with agitation (180 rpm) to induce the expression of I-*Sce*I and to cause the double-strand break in the genome and promote the second step of recombination between the two mutant alleles. A sample of these cultures was streaked on LB-Cm (or Sp) agar plates and incubated o/n to isolate individual colonies, which were replicated in LB-Cm (or Sp) and LB-Amp-Cm (or Sp) agar plates to identify Amp-sensitive colonies, which were the EPEC mutant strains without the Amp.FRT cassette. Amp-sensitive mutant were screened by PCR using specific oligonucleotides listed in Table 4.

Plasmid pACBSR was cured from the final strains by growth in liquid LB (5 passages approximately) and streaking on LB agar plates. Individual colonies were replicated in LB and LB-Cm (or Sp) plates to screen for Cm (or Sp)-sensitive colonies.

5. SDS-PAGE and Western blot

Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were performed following standard methods (Ausubel F.M. 2002) using the Miniprotean III system (Bio-Rad). Proteins separated by SDS-PAGE were either subjected to Coomassie Blue R-250 (Bio-Rad) staining or Western blot. For the latter, the proteins were transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore) using semi-dry and wet electrophoresis (Bio-Rad), as previously described (Ausubel F.M. 2002). Antibodies employed for Western blot are described in Table 5. Membranes were developed by chemiluminiscence using the Clarity Western ECL Substrate kit (Bio-Rad), SuperSignal West Femto (Thermo Scientific) or a mixture of 100 mM Tris-HCl (pH 8.0) containing 1.25 mM luminol (Sigma), 0.22 mM cumaric acid (Sigma), and 0.0075% (v/v) H2O2 (Sigma). The membranes were then developed by exposure to X-ray films (Agfa) or with a Fuji LAS 3000 image when the signal was quantified.

For the analysis of the secretion of T3SS components, EPEC strains were grown in DMEM and bacteria were harvested from 1 ml aliquots of the induced cultures by centrifugation (14000 g, 5 min). To obtain whole-cell protein extracts, the cells were resuspended in 400 µl of phosphate-buffered saline (PBS), mixed with 100 µl of 5X SDS-PAGE sample buffer and boiled for 10 min, and then protein were separated by SDS-PAGE and analyzed by Western blot. For the analysis of the T3-secreted proteins, the bacterial cultures were centrifuged at 4 ºC in 50 ml Falcon tubes (BD Biosciences) during 5 min (4000 rpm). This was done twice to ensure removal of most bacterial cells. Later 1 ml of the culture supernatants was chilled on ice and incubated 60 min with trichloroacetic acid (TCA 20% w/v; Merck) for

precipitation. After cold centrifugation (14000g, 15 min), TCA-precipitated protein pellets were rinsed with cold acetone (-20 °C) and resuspended in 30 µl of SDS-PAGE sample buffer for Coomassie staining or Silver staining.

To evaluate the amount p65 protein into HeLa cells after infection with EPEC strains. HeLa cells were scraped from the wells using 500 µl of lysis buffer 1X. To prepare 7 ml of lysis buffer 1X we mixed: 175 µl of Tetrasodium Diphosphate Decahydrate-NaPPi 250mM, 25 µl of Protease Inhibitor Cocktail (P8340, Sigma), 1 ml of lysis buffer 7X and 5.66 ml of H2O. The lysis buffer 7X is: Tris base 350mM (pH 7.4), NaCl 1.05 M, EDTA 14 mM, and NP-40 7%. This buffer was kept in aliquots of 1 ml at -20 °C until use. The lysed cell samples were recovered in a 1.5 ml eppendorf tube and incubated during 30 min at 4 °C with rotation. Then samples were centrifuged (14000g, 15 min) and 400 µl of the supernatant were mixed with 5X SDS-PAGE sample buffer and then protein were separated by SDS-PAGE and analyzed by Western blot.

Table 5. Antibodies used for Western Blots

Protein	Primary Antibody	Secondary Antibody
EspB	Rabbit polyclonal anti-EspB (1:2000)	Protein A-HRP (Life Technologies, 1:5000)
EscC	Rabbit polyclonal anti-EscC (1:1000)	Protein A-HRP (Life Technologies, 1:5000)
EscJ	Rabbit polyclonal anti-EscJ (1:5000)	Protein A-HRP (Life Technologies, 1:5000)
EscD	Rabbit polyclonal anti-EscD (1:1000)	Protein A-HRP (Life Technologies, 1:5000)
GroEL	anti-GroEL mAb-POD (Sigma, 1:5000)	
Tir	Mouse monoclonal anti-Tir (1:1000)	Goat anti-mouse HRP conjugated (Jackson ImmunoResearch, 1:5000)
NF-κB p-65	Rabbit polyclonal anti NFκB p-65 SC-109 (Santa Cruz, 1:1000)	Goat anti-rabbit HRP conjugated (Jackson ImmunoResearch, 1:5000)
Tubulin	Mouse monoclonal anti-alpha Tubulin clone DM1A (Sigma, 1:1000)	Goat anti-mouse HRP conjugated (Jackson ImmunoResearch, 1:10000)
Intimin	Rabbit polyclonal anti-intimin-280 (1:5000)	Protein A-HRP (Life Technologies, 1:5000)

6. Mammalian cell cultures *in vitro*

The human cell lines HeLa (ATCC, CCL-2) were routinely grown as monolayers in DMEM, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma) and 2 mM glutamine, at 37 °C with 5% CO_2. During experiments of infections to evaluate individual injection of NleC and Tir by the EPEC-strains, the HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS; Sigma), 2 mM glutamine and 1% of MEM non-essential amino acid solution 100X (Sigma). Swiss 3T3 mouse fibroblasts (ATCC; CCL-92) were grown as monolayer in DMEM-high glucose, with 4500 mg glucose/l (D5671; Sigma) supplemented with 10% of heat-inactivated fetal calf serum (FCS; Sigma), 2 mM glutamine and 1X of MEM non-essential amino acid solution 100X (Sigma).

6.1 EPEC infections of mammalian cells cultured *in vitro*

HeLa cells were seeded approximately 24 h before to the infection to obtain ~90% cell confluency i.e., 1.8 - 2.0 x 10^5 cells per well in 24-well tissue culture plates (Falcon). HeLa cells were washed once with pre-heated serum-free DMEM 2 h before the infection with EPEC strains. Multiplicity of infection (MOI) and time of infection are described in individual experiments.

During infections to evaluate individual translocation of NleC and infections to evaluate the translocation of Tir by EPEC-strains, 2.4 x 10^5 HeLa cells/well were seeded 48 h before to the infection to obtain cell confluency between 70% - 80% (8.4 and 9.6 x 10^5 cells) in 6-well tissue culture plates (Falcon). HeLa cells were washed three times with sterile pre-warmed PBS (Sigma) and serum-free DMEM was added to starve the cells 2 h previous to the infection. Infections were done with 1.5 ml of EPEC strains and 3 ml of EPEC0 strains cultures, induced during 3 h previous to the infection. Cells were infected 1 h and then washed three times with PBS and incubated for additional 3 h with 200 µg/ml of gentamicin in DMEM. Cells were washed with PBS to remove the unbound bacteria and the cell extracts were analyzed by Western blot. At the beginning of the infection plates were centrifuged to synchronize the infection (500 g, 5 min).

To analyze the phenotype of filopodia formation, infection with EPEC strain was done in Swiss 3T3 cells because these cells have more active actin dynamic than other cell lines. The Swiss 3T3 cells were seeded 48 h before to the infection to obtain cell confluence between 60% and 70% (1.2 and 1.4 x 10^5) cells in 24 well tissue culture plates (Falcon). Swiss 3T3 cells were washed three times with sterile

pre-warmed PBS (Sigma) and serum-free DMEM was added to starve the cells 2 h previous to the infection. Infections were done with 500 µl of EPEC strains induced during 3 h previous to the infection. The plates were centrifuged to synchronize the infection (500 g, 5 min) and the infection was incubated during additional 5 min.

6.2 β-Lactamase translocation assay

The previously described assay to quantify β-lactamase (Bla) translocation was adapted for EPEC-wt and EPEC derived mutant strains (Charpentier and Oswald 2004). HeLa cells were seeded in a 96-well opaque plate (Nunc) 48 h previous to the infection to obtain a cell confluence of 4x104 cell/well, at 37°C with 5% CO_2. The culture medium of the cells was changed 2 h before to the infection by serum-free DMEM. HeLa cells were infected with the induced EPEC strains with a MOI 100:1, after 30 min of infection 1 mM of IPTG was added, and the infections were maintained during 1 extra hour under these conditions. Then cells were washed twice with preheated DMEM and cover with 100 µl of HBSS and 20 µl of 6X CCF2/AM solution (CCF2/AM final concentration 1 µM, Invitrogen). Samples were incubated for 1 h in the dark at a room temperature (RT) and plates were read in a SpectraMax M2 fluorometer (Molecular Devices) with a filter set 450/520 nm.

6.3 LDH-release assay

HeLa cells were seeded in 24 well plates and washed twice with preheated color-free (without phenol-red) DMEM (Sigma) 2 h previous to the infection. HeLa cells were infected with EPEC induced strains using a MOI 200:1 during 1.5 h and then cells were washed twice with DMEM color-free and incubated with DMEM color-free during additional 1.5 h. Plates were centrifuged (250 g, 10 min) and 500 µl of supernatants were harvested and maintained in 1.5 ml eppendorf tubes at 4°C. To obtain the total LDH release from the cells, uninfected cells were treated with Triton X-100 (1%, 10 min). LDH release was measured mixing 100 µl of the culture supernatants and 100 µl of the reaction mixture of the LDH Cytotoxicity Detection Kit (Clontech). The percentage of LDH release is calculated as the amount measured in the supernatant divided by the total amount of LDH in the cells. Absorbances of samples were read at 490 nm using iMark ELISA plate reader (Bio-Rad).

6.4 Immunofluorescence microscopy

Infected HeLa cell cultures, grown on coverslips in 24-well plates, were washed three times with 1 ml/well of PBS (sigma), fixed with 4% (w/v) paraformaldehyde (in PBS, 20 min, RT) and washed again with PBS three times. Cells were permeabilized by incubation in a solution of 0.1% (v/v) of saponin (Sigma) in PBS for 10 min and washed with PBS three times. The primary antibodies were added in PBS-10% goat serum (Sigma) and incubated 60 min at RT. Coverslips were washed three times with PBS. Then coverslips were incubated 45 min with the conjugated secondary antibodies in PBS-10% goat serum, together with Phalloidin TRITC (1:500; Sigma) and DAPI (1:1000; Sigma) to label F-actin and DNA, respectively. Coverslips were washed 3 times with PBS after incubation and 4 μl of ProLong Gold anti-fade reagent (Life technologies) was used to mount coverslips and they were dried o/n. Samples were observed in a SP5 confocal microscope (Leica) using the 100X objective or a fluorescence Olympus microscope (BX61) using the 40X and 60X objective. Reagents, antibodies and fluorophores used in microscopy is described in Table 6.

Infected Swiss 3T3 cell cultures, grown on coverslips in 24-well plates, were washed three times with 1 ml/well of PBS (sigma), fixed with 4% (w/v) paraformaldehyde (in PBS, 20 min, RT) and washed again with PBS three times. Fixed monolayers were treated with 50 mM NH4Cl (10 min, RT) for formaldehyde function neutralization. Cells were permeabilized by incubation in a solution of 0.2% (v/v) of Triton X-100 in PBS (4 min, RT) and washed with PBS two times. Then cell were treated with PBS-0.2% BSA (10 min, RT). The primary antibodies were added in PBS-0.2% BSA and incubated 60 min at RT. Coverslips were washed twice with PBS and then treated with PBS-0.2% BSA during 5 min. Then coverslips were incubated 45 min with the conjugated secondary antibodies in PBS-0.2% BSA together with Oregon-green Phaloidine (1:100, Invitrogen) to label bacteria and actin respectively. Coverslips were washed 3 times with PBS after incubation and were mounted with one drop of prolong reagent and were dried o/n. They were then observed at the Zeiss Axio imager microscope. The projections of the images were done with AxioVision LE re. Software (Zeiss). The list of antibodies used is described in Table 6.

After inducing EPEC strains in serum-free DMEM during 2.5 h, bacteria was collected and washed with PBS and resuspended to a 0.5 OD_{600} and keep on ice. Coverslips were treated previously with 50 μl of poly-L-Lysine solution (ChemCruz) at RT, after 30 min the poly-L-Lysine was removed. Then 10 µl of the cell solution were added over the coverslips and incubated during 30 min at RT until it is dry, use the

chamber. Coverslips were washed with PBS to remove unbound bacteria. Add 300 µl PFA 4% during 20 min. Coverslips were washed 3 times with PBS and later the bacteria were stained with antibodies anti intimin-280 (Table 6).

Table 6. Reagents and antibodies used for fluorescence microscopy

Antigen	Primary reagent/antibody	Secondary antibody
EPEC	Rabbit polyclonal anti-intimin-280 (1:500) Rabbit polyclonal anti O127 (1:100)	Goat anti-rabbit-AL-EXA488 (Life technologies, 1:500) Donkey anti-rabbit-AL-EXA488 (Jackson ImmunoResearch, 1:100)
Actin	Phalloidin-TRITC (1:500) Oregon-green Phalloidin (1:500)	
DNA	DAPI (4',6-diamidino-2-phenilindole, 1:1000, Life technologies)	
Vinculin	Monoclonal mouse anti-Vinculin clone hVIN-1 (1:400, Sigma)	Goat anti-mouse-AL-EXA488 (Life technologies, 1:500)

7. EPEC infections of human intestinal biopsies cultured *in vitro*

Duodenal mucosal biopsies taken with informed consent and ethical approval were maintained in organ culture and infected for 8 h with o/n LB cultures of EPEC-wt and EPEC mutant strains, as described previously in (Knutton, Lloyd et al. 1987) .

7.1 Samples for Scanning Electron Microscope (SEM)

Infected intestinal biopsies were thoroughly washed and fixed with 3% of glutaraldehyde and processed for scanning electron microscopy (SEM) (Knutton 1995) and examined in a scanning electron microscope Jeol JSM-6390.

8. RT-PCR to evaluate *efa1/lifA*-like and *nleE2* gene expression

RNA was extracted from the EPEC strains and reversed transcribed by RT-PCR as described in(Hemrajani, Marches et al. 2008, Pallett, Berger et al. 2014). The primers used for the RT-PCR are listed in Table 3.

RESULTS

1. Markerless gene deletion strategy in EPEC O127:H6 E2348/69

We decided to use in our research the prototype EPEC O127:H6 strain E2348/69 which has been widely studied to characterize the effectors of the T3SS using conventional genetics, genomic and proteomic analysis, providing a good knowledge of the whole repertoire of effectors encoded in this EPEC strain (Kenny, DeVinney et al. 1997, Iguchi, Thomson et al. 2009, Deng, Yu et al. 2012). We wanted to use a strategy for deletion and integration of genes in EPEC genome without leaving any marker or "scars" in the chromosome. The marker-less gene replacement strategy chosen is based in the homologous recombination of a mutant allele, cloned in a suicide plasmid, and flanked by the restriction sites for the meganuclease I-*Sce*I (Figure 9).

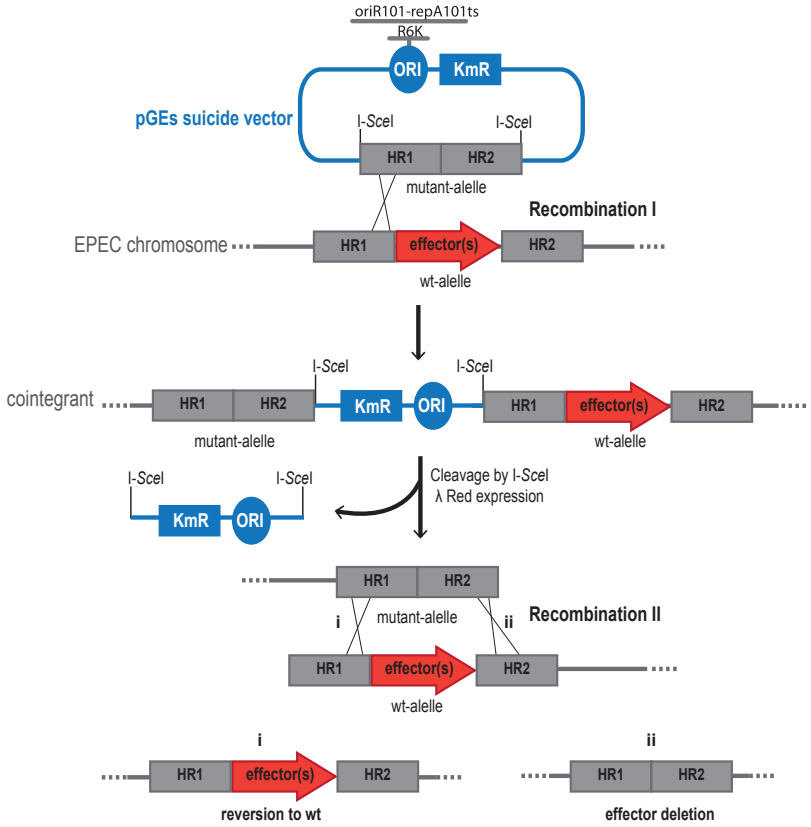

Figure 9. Markerless gene deletion strategy. Deletions were carried out with suicide plasmids pGEs with mutant alleles. pGE contains an R6K replication origin (π protein dependent) and pGEts contains a thermo-sensitive replication origin ori101 (replicates at 30ºC and can not replicate 37-42ºC). The lacks of π protein in EPEC or the growth at non-permissive temperature induce integration of pGEs and co-integrants of the wt and mutant alleles are obtained. Co-integrants are identified by the Km resistance. Expression of the I-*Sce*I in vivo from a helper plasmid induces a double strand brake (DSB) and after a second homologous recombination, the chromosome can revert to the wt or to the mutant allele.

Following the expression *in vivo* of I-*Sce*I, which induces cleavage of the I-*Sce*I sites generating double-strand break (DSB) in the chromosome of the co-integrant strain that stimulates a repair mechanism driven by a second homologous recombination resulting in either mutant or wt allele being maintained in the chromosome (Posfai, Kolisnychenko et al. 1999). Genome modifications were done with this marker-less gene replacement strategy with minor modifications, like the expression of λ Red proteins to assist recombination (Pinero-Lambea, Bodelon et al. 2014).

1.1 Deletion of the ATPase *escN* and the negative regulator *grlR*

With the aim to evaluate the applicability in EPEC of the marker-less gene deletion strategy, we tested the deletion of *escN*, which is the ATPase that energizes the protein secretion through the T3SS injectisome. We generated a suicide plasmid, pGEΔ*escN*, with a mutant allele for deletion of *escN*. After integration of this plasmid and resolution of the co-integrants we selected the strain that resolved to the mutant Δ*escN* allele. To appraise the behavior of EPECΔ*escN* we evaluated the pattern of proteins secreted into the extracellular medium compare to EPECwt strain (Figure 10). In EPEC, the LEE genes are expressed at 37 °C in DMEM and repressed in LB medium (Kenny, Abe et al. 1997). After growth of EPECwt in DMEM, we could observe the EspA, EspB and EspD translocators in the extracellular medium. We could also observed EspC autotransporter, which is secreted independently of the

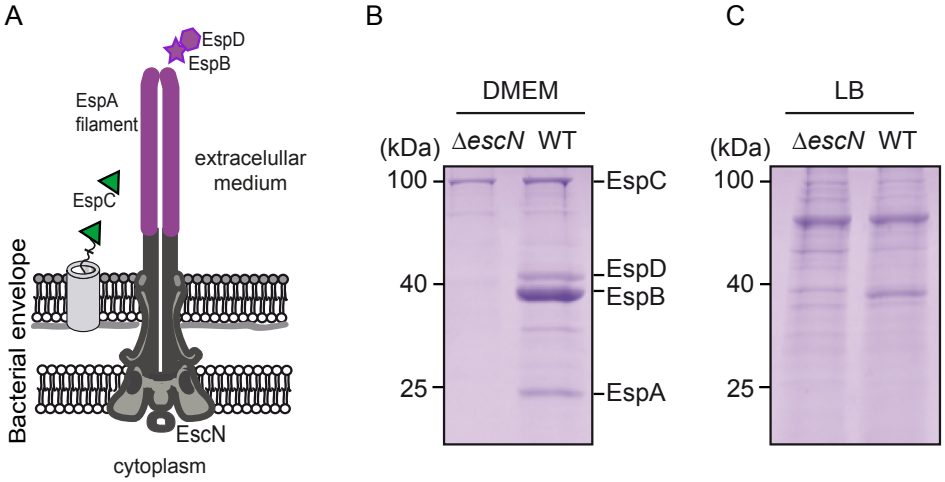

Figure 10. Proteins secreted by EPEC. (A) Schematic representation of the T3SS injectisome, indicating the filament of EspA, the translocator proteins EspB and EspD, and the ATPase EscN. Secretion of the autotransporter EspC is also shown. **(B)** Coomassie staining of the proteins in the extracellular media of EPECΔ*escN* and EPEC-wt strains grown 4h at 37°C in DMEM or LB. The translocators EspABD and the autotransporter EspC proteins are labeled. Molecular standards mass proteins are shown in kDa.

T3SS. In contrast, EPECΔ*escN* bacteria, grown under the same conditions, do not secrete the translocator proteins to the extracellular medium, while EspC is still found because the disruption of the T3SS does not affect its secretion.

Next, we tested the marker-less deletion of *grlR*, which encodes a negative regulator of Ler and indirectly of LEE genes since Ler is a positive regulator of all other LEE promoters and of EspC, which is located outside the LEE (Kaper, Nataro et al. 2004). It has been reported that deletion of *grlR* in EPEC derepress LEE island and induces its expression in LB (Garcia-Angulo, Martinez-Santos et al. 2012). We generated the deletion of the *grlR* in EPECwt strain and compared the pattern of the extracellular proteins between the two strains grown in LB. Contrary to the parental WT strain, EPECΔ*grlR* strain was able to secrete the translocator proteins EspABD and the autotransporter EspC to the extracellular medium even under the repressing conditions in LB (Figure 11).

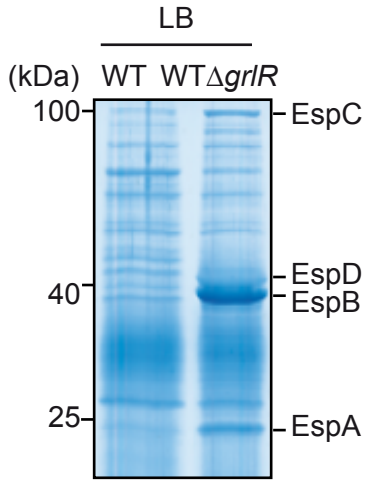

Figure 11. Secreted proteins in LB by EPECwt and EPECΔ*grlR*. Coomassie staining of the proteins found in the extracellular media of cultures of EPECwt and EPECΔ*grlR* grown in LB at 37 °C. The translocators EspABD and the autotransporter EspC are labelled. Molecular mass standards are shown in kDa.

2. Generation of the effector mutant EPEC strains

Once we confirmed that the marker-less gene deletion strategy was useful to manipulate EPEC genome, we designed a set of suicide plasmids with R6K- or pSC101-thermosensitive origin of replication for the deletion of all effectors genes known in the genome of EPEC strain E2348/69. The strategy was done to delete the effector genes from the start to the stop codon of individual ORFs of gene effectors or

clusters of gene effectors, maintaining the endogenous transcriptional promoters and terminator signals of these genes and leaving no antibiotic resistance markers or scars in the chromosome.

2.1 Deletion of LEE encoded effector genes except *espZ* and *tir*

We started with the deletions of LEE encoded effector genes (Figure 12). Since all the genes responsible for the assembly of the T3SS injectisome are localized in the LEE island, we wanted to confirm that these deletions do not affect T3SS. We made the sequential deletion of the following effector genes: *map, espG, espF* and *espH*. We decided not to delete *espZ* and *tir* at this stage. In the case of *espZ* it was due for its reported ability to control the effector translocation through the T3SS (Berger, Crepin et al. 2012), which may be necessary to provide a physiological amount of translocation of the remaining effectors proteins. In the case of Tir its translocation and insertion into the enterocyte plasma membrane and its interaction with intimin to trigger actin polymerization beneath the adherent bacterium could be useful to evaluate the functionality of the T3SS injectisome (Kenny, DeVinney et al. 1997). After deletion of *map*, *espG*, *espF* and *espH* we obtained the strain called EPEC9 Table 1.

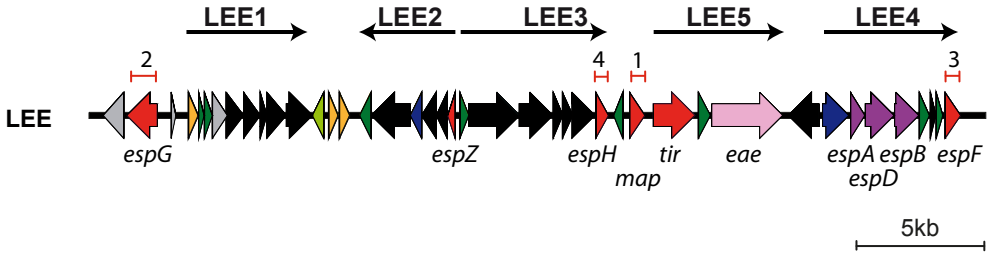

Figure 12. LEE effector genes. Representation of the LEE island. Effector genes are labeled in red. The order of deletion is numbered: deletion 1, 2, 3, 4 are for map, espG, espF and espH respectively. The espZ and tir genes were not deleted at this stage given their role in regulating T3SS translocation and actin-pedestal formation. Scale of 5kb is indicated at the bottom.

2.2 Deletion of non-LEE encoded effector genes

Next we initiated the deletion of the non-LEE effector genes. Since these effector genes are in clusters in integrative elements (IEs) and prophages (PPs) in the genome of EPEC Figure 13, the HR were selected to flank these clusters in order to delete simultaneously several effector genes. The order of deletion followed was: IE5 (*espG* and *espC*), IE6 (*espL*, *nleB1*, *nleE1*, *efa1/lifA*) and IE2 (*espL*, nleB*,*

nleE2, efa1/lifA-like). The resulting strain was called EPEC6. Although EspC is not an effector protein, we deleted *espC* together with *espG* in the IE5. EspC is an autotransporter protein, which was reported to cause cytopathic effect on epithelial cells and to be internalized in the host cell in a T3SS-dependent manner (Vidal and Navarro-Garcia 2006, Vidal and Navarro-Garcia 2008). EspC cytotoxicity is characterized by cell rounding and cell detachment phenotypes, which are induced by cytoskeletal and focal adhesion disruption and depends on its internalization and functional serine protease motif (Navarro-Garcia, Canizalez-Roman et al. 2004, Navarro-Garcia, Serapio-Palacios et al. 2014) .Additionally, it has been reported that EspC is involved in EPEC-mediated cell death and induces apoptosis and necrosis (Serapio-Palacios and Navarro-Garcia 2016). Lastly, we deleted the effector genes present in PPs following this order: PP2 (*nleH1*, *cif*, *espJ*), PP3 (*nleJ*), PP4 (*nleG*, *nleB*, *nleC*, *nleH*, *nleD*) and PP6 (*nleA*/*espI*, *nleH2*, *nleF*, *espO*). After these deletions we obtained the strain called EPEC2. As explained later the functionality of the T3SS was evaluated after every deletion.

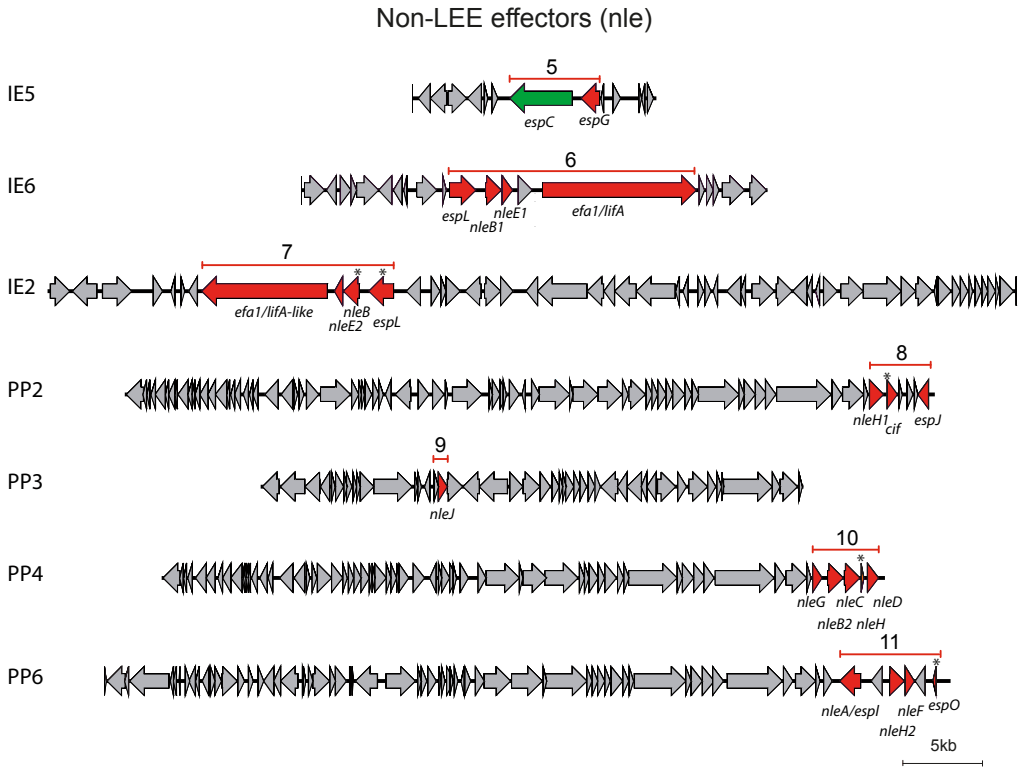

Figure 13. Non-LEE effector genes in the chromosome of EPEC E2348/69. Effector genes located outside the LEE are localized in integrative elements (IE) and prophages (PP). Effector genes are labeled in red. The red bars indicate the cluster of genes deleted. Pseudogenes are specified with asterisk. The order of deletion is numbered. Scale of 5 kb is indicated at the bottom.

2.3 Deletion of *espZ* and *tir*

We then focused on the deletion of *espZ* and *tir*. Considering that *espZ* was the first gene of the *LEE2* operon, it was possible that its deletion could affect the expression of downstream genes in the operon and, as a consequence, of the T3SS injectisome. We first deleted the ORF of *espZ* in an EPECwt strain, from the start codon to the stop codon, generating a mutant strain with *escI* as the first gene of the *LEE2* operon. In this mutant, upstream of *escI* is localized its own RBS and the RBS of *espZ* (Figure 14A). We observed reduction in the secretion of the translocator proteins EspABD in this EPECΔespZ-1 mutant strain (Figure 14B). Thus, we designed a different plasmid for deletion of *espZ* and its RBS. With this new construct the *LEE2* starts with *escI* and its RBS (Figure 14A) and the mutant EPECΔ*espZ*-2 has a level of T3SS secretion similar to the EPECwt strain (Figure 14B). Thus, we used this second construction to delete *espZ* in the EPEC2 strain, generating EPEC1, which only carries *tir* effector. Next we deleted *tir* generating and EPEC0 strain devoid of all known T3 secreted effector genes, see all the generated strains in Table 1.

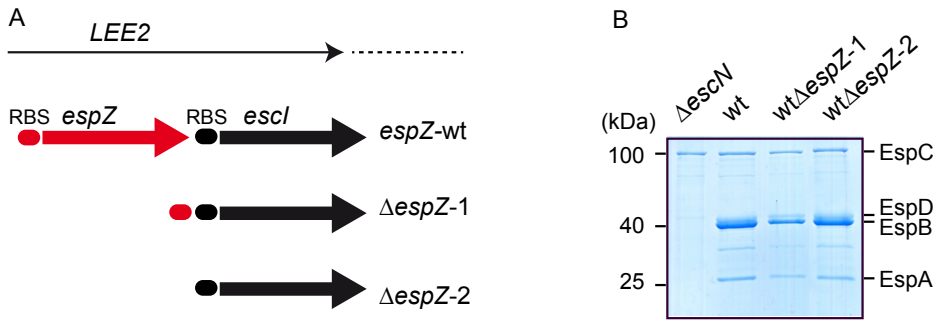

Figure 14. Extracellular proteins secreted by EPECwt and ΔespZ mutants. (A) Schematic representation of gene organization of *espZ* and *escI* with their RBS in the LEE2 operon of wt strain and Δ*espZ*-1 and Δ*espZ*-2 mutants. **(B)** Coomassie staining of proteins secreted in the extracellular media of EPECΔ*escN*, EPECwt, EPECΔ*espZ*-1 and EPECΔ*espZ*-2 strains grown 4 h in DMEM at 37 °C. The translocators EspABD and the autotransporter EspC are labeled. Molecular standards mass proteins are shown in kDa.

2.4 Confirmation of gene deletion and genome sequencing

During generation of each mutant strain we tested by PCR the parental and mutant strains to confirm introduction of the expected deletion (see Table 4 for primers used). After all deletions, we performed a PCR to confirm all deletions in EPEC0 strain. In Figure 15 we show the final confirmation of all deletion in EPEC0 compared to EPECwt.

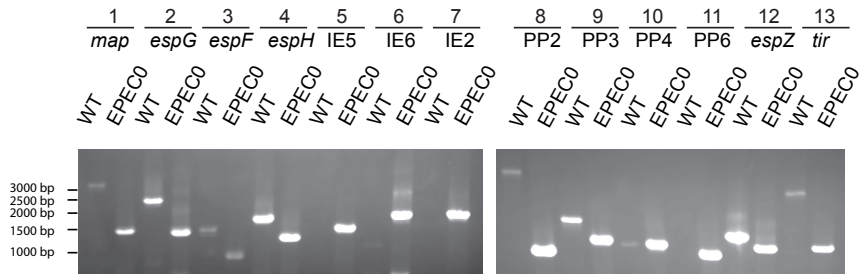

Figure 15. PCRs to confirm deletion of effectors in EPEC0. Electrophoresis gel of PCR products amplified from EPECwt and EPEC0. The amplicons corresponding to LEE effector genes or clusters deleted are indicated in the top of the gel. The order of the deletions from 1 to 13 is numbered. DNA ladder is labeled on the left.

We also performed whole genome sequencing of the parental EPECwt strain and the EPEC1 strain, which maintains Tir translocation into HeLa cells (see below). Comparison of these genomes confirmed that the only differences between them were the designed deletions of effector genes (Table 7).

Table 7. Positions of genome deletions respect to EPECwt

Effector(s) deleted in EPEC1	Deletions		Size of deletion
	Start position	End position	
espG	829165	830361	1197
espZ	841896	842221	326
espH	847765	848295	531
map	849134	849745	612
espF	861663	862283	621
IE6	1549768	1568027	18260
IE5	1879645	1885304	5660
PP6	3411058	3417094	6037
IE2	3705811	3720184	14374
PP4	3761499	3765510	4012
PP3	3985969	3986516	548
PP2	4078295	4082262	3968

2.5 Growth and viability of effector mutant EPEC strains

The growth of EPEC2, EPEC1 and EPEC0 and the parental EPECwt was compared in liquid LB cultures, showing an identical growth curve (Figure 16A). We also measured the viability (CFU/OD) of EPEC2, EPEC1 and EPEC0 and the parental EPECwt in DEMEM after 2.5h, serial dilutions were plated in LB plates in triplicate

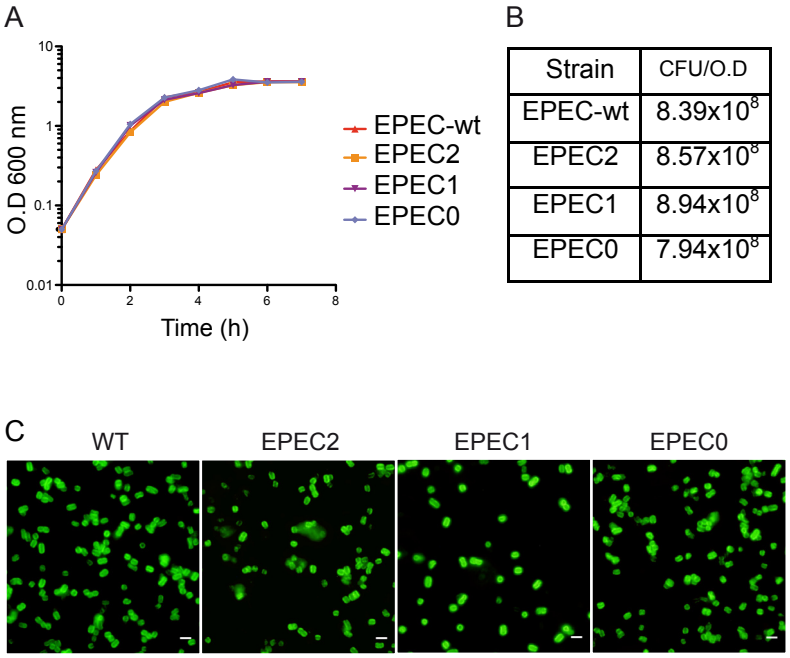

Figure 16. Growth curve, viability and morphology of EPEC effector mutant strains. (A) EPECwt, EPEC2, EPEC1 and EPEC0 were grown in LB and we measured the optical density (O.D) at 600nm at the indicated times points. (B) CFU/OD of the indicated strains (EPECwt, EPEC2, EPEC1 and EPEC0) grown in DMEM and plated in LB-agar.(C)Immunofluorescence microscopy of EPECwt, EPEC2, EPEC1 and EPEC0. EPEC strains are stained with anti-intimin-280 polyclonal serum (green). Scale bar 2 µm.

to count the number of CFU per O.D. All strains had a similar viability (Figure 16B). We also inspected bacteria from DMEM cultures by immunofluorescence microscopy after staining with anti-intimin-280, which revealed a similar size and morphology of all bacterial strains (Figure 15C). Therefore the deletion of all effectors in the genome of EPEC did not affect growth, viability and morphology of bacteria.

3. Evaluation of T3SS injectisome functionality in effector mutant EPEC strains

3.1 Secreted protein by effector mutant EPEC strains.

To confirm the functionality of the T3SS after gene deletions we first analyzed the proteins secreted in the extracellular medium of effector mutant EPEC strains. Bacteria from parental and effector deletion strains were grown in DMEM at 37°C and the presence of secreted EspABD translocator proteins analyzed by SDS-PAGE. EPECΔ*escN* was used as a negative control (Figure 17). We found that secretion

of translocator protein EspABD was unaffected in the effector mutant strains. As expected EspC was absent after IE5 deletion in EPEC8.

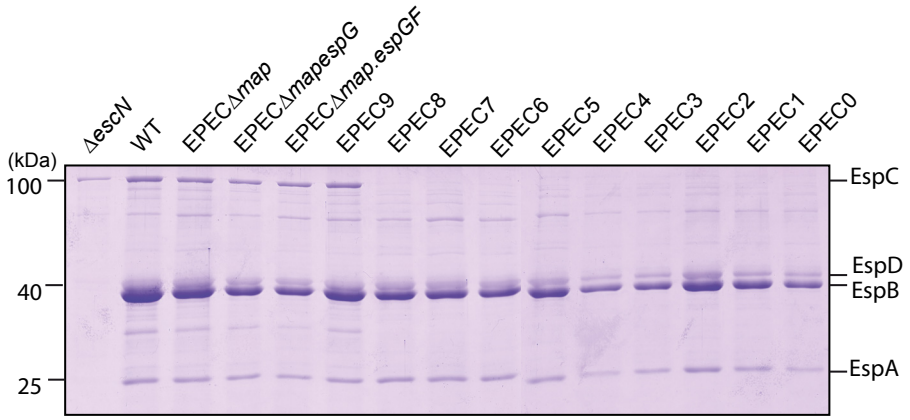

Figure 17. Proteins secreted by EPECwt and derived mutant strains. Coomassie staining of proteins found in the extracellular medium, after 4 h of growth in DMEM at 37ºC. The translocators EspABD and the autotransporter EspC are labeled. Molecular standards mass proteins are shown in kDa.

3.2 Expression of injectisome-proteins in effector mutant EPEC strains

We also evaluated the expression of the structural proteins EscC, EscJ, EscD and the translocator protein EspB in all mutant strains by analyzing whole cell protein extracts by Western blot (Figure 18). All the effectors mutant strains have equal expression of the injectisome-proteins compared to EPECwt. Therefore, deletion

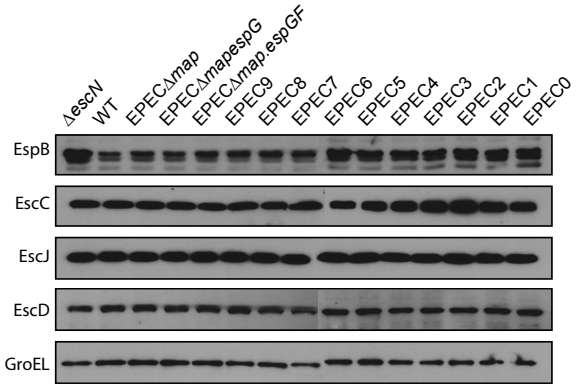

Figure 18. Expression of injectisome-proteins in EPEC effector mutant strains. Bacteria from the indicated strains were grown in DMEM at 37 ºC during 4 h and whole-cell protein extracts analyzed by Western blot. Rabbit polyclonal antibodies were used to detect EscC, EscJ, EscD structural proteins and EspB translocator protein. Detection of cytoplasmic GroEL was used as a loading control.

of effector genes did not affect the expression of injectisomes. These results, along with the secretion of translocator proteins into the extracellular medium, indicated the correct assembly of the T3SS injectisomes in the effector mutant strains generated.

3.3. Infection of HeLa cells by effector mutant EPEC strains.

When EPEC bacteria adhere *in vitro* to cultured cells there is accumulation of actin filaments in the cytoplasm beneath the adherent bacteria, due to a signal cascade generated by Intimin-Tir interaction (Kenny, DeVinney et al. 1997). This recruitment of actin beneath the attached bacteria induces pedestals-like structures similar to those observed *in vivo* in A/E lesions (Knutton, Baldwin et al. 1989). Thus, to investigate whether effector mutant EPEC strains were able to translocate Tir into the cytoplasm of the cells and induce the actin accumulation underneath the adherent bacteria, we infected HeLa cells (Figure 19) with EPECwt and the derived

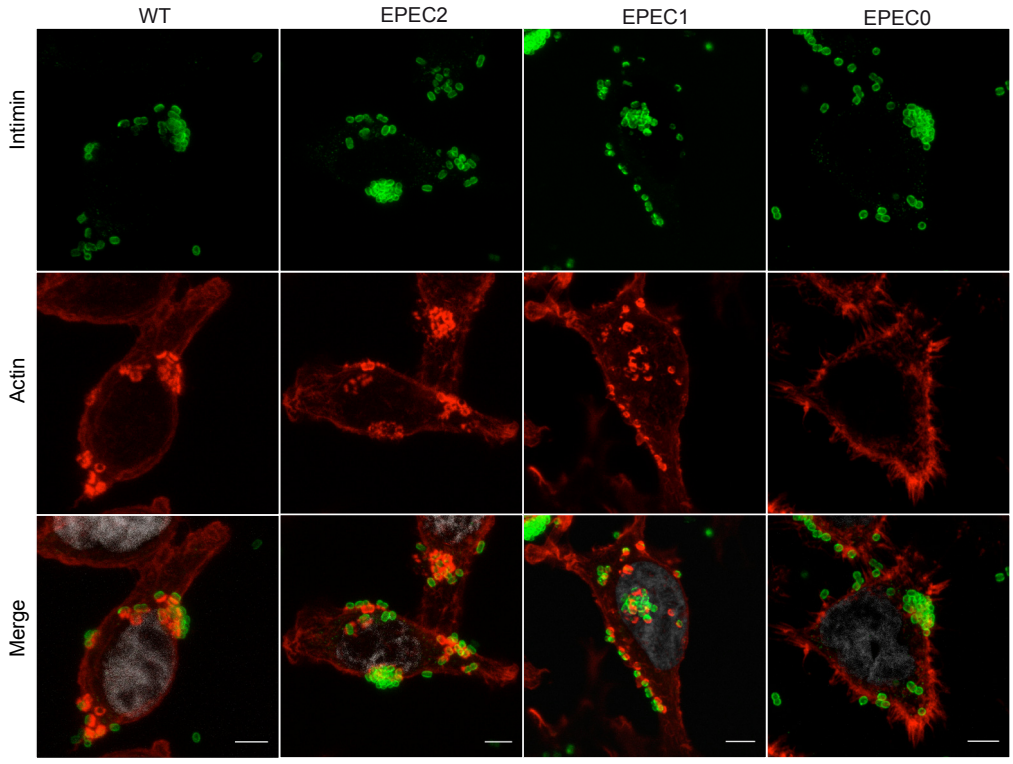

Figure 19. Infection of HeLa cells with EPECwt and effector mutant EPEC strains. Immunofluorescence confocal microscopy of HeLa cells infected 1.5 h using a MOI 200:1 with EPECwt, EPEC2, EPEC1 and EPEC0 strains. EPEC is labeled with anti-intimin-280 serum (green), actin is labeled with TRITC phalloidin (red) and cell nuclei are labeled with DAPI (gray). Actin polymerization beneath the adherent bacteria is observed in EPECwt, EPEC2 and EPEC1. Scale bar 5 µm.

effector mutant strains. Cultured HeLa cells were infected during 1.5 h and then fixed and stained for immunofluorescence microscopy. Actin accumulation was present in EPECwt, EPEC2 and EPEC1 but, as expected not in EPEC0 due to absent of *tir*. All other mutant strains also produced actin polymerization upon infection (data not shown). These results confirm that the individual translocation of Tir by EPEC1 is sufficient to induce actin accumulation during *in vitro* infection of HeLa cells. We also observed the typical microcolonies of the EPEC infection in all mutant strains, formed by the interaction between the bacteria through the BFP (Ramer, Bieber et al. 1996), indicating that this EPEC phenotype was not disrupted.

3.4 Deletions of Intimin in EPEC2 and EPEC1.

Intimin is necessary to induce actin polymerization underneath the attached bacteria (Jerse, Yu et al. 1990). We deleted *eae*, the gene encoding intimin, in EPEC2 and EPEC1 to confirm that the actin accumulation seen underneath these bacteria depends on the interaction of intimin with translocated Tir. We analyzed the extracellular secreted proteins of the EPEC2 and EPEC1 Δ*eae* mutant strains compared to EPECwt (Figure 20A). Western blot of the bacterial lysates proved deletion of *eae* in EPEC2 and EPEC1 Δ*eae* strains (Figure 20A). We infected HeLa cells with EPEC2 and EPEC1 and the isogenic Δ*eae* mutant strains, to confirm formation of actin-pedestals by these strains. Whereas EPEC2 and EPEC1 bacteria infect HeLa cells and induce actin polymerization underneath the microcolonies, the isogenic Δ*eae* mutant strains do not induce actin polymerization underneath the attached bacteria. Also, microcolonies of Δ*eae* mutants are smaller than those of parental strains (Figure 20B). These results confirm that the effector mutant strains induce actin pedestals behind the attached bacteria that depend on the interaction of intimin and translocated Tir.

3.5. Quantification of β-lactamase translocation into HeLa cells by effector mutant EPEC strains

We tested the capability of the EPEC effector mutant strains for protein translocation into HeLa cells using the reporter enzyme β-lactamase (Bla) (Figure 21). For this aim, we used pEspF$_{1\text{-}20\text{-}}$Bla plasmid, which has the N-terminal 20 amino acids of the EspF effector protein fusion to Bla to drive its T3S-dependent translocation (Charpentier and Oswald 2004, Blanco-Toribio, Muyldermans et al. 2010). We tested translocation of EspF$_{1\text{-}20\text{-}}$Bla by EPECwt, EPEC2, EPEC1 and EPEC0. As a negative control we used EPECΔ*escN* with the pEspF$_{1\text{-}20\text{-}}$Bla and the EPECwt harboring pCX340. The pCX340 plasmid has the Bla reporter without

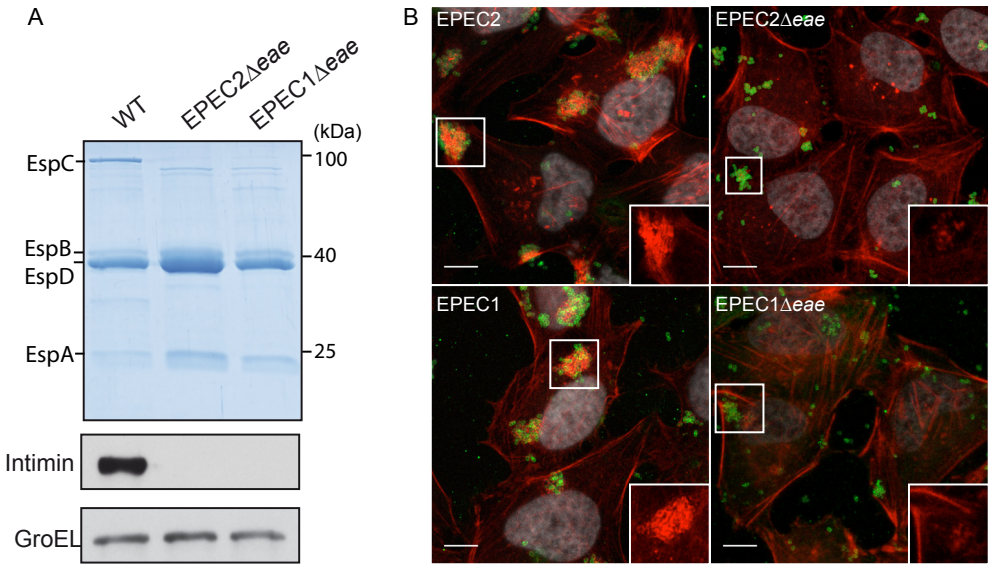

Figure 20. Deletion of eae in EPEC2 and EPEC1 strains. (A) Coomassie staining of proteins secreted in the extracellular media. EPECwt, EPEC2Δ*eae* and EPEC1Δeae were grown in DMEM at 37°C during 4 h. The translocators EspABD and the autotransporter EspC are labeled. Protein standards for SDS-PAGE in kilodaltons (kDa) are shown. Western blot of bacterial lysates: detected with rabbit polyclonal anti-intimin-280, GroEL (as a loading control). **(B)** Immunofluorescence confocal microscopy of HeLa cells infected 1.5 h with EPEC2, EPEC2Δ*eae*, EPEC1 and EPEC1Δ*eae* (MOI 200:1). EPEC is labeled with anti-intimin-280 serum (green), actin is labeled with TRITC phalloidin (red) and cell nuclei are labeled with DAPI (gray). Scale bar 5 µm

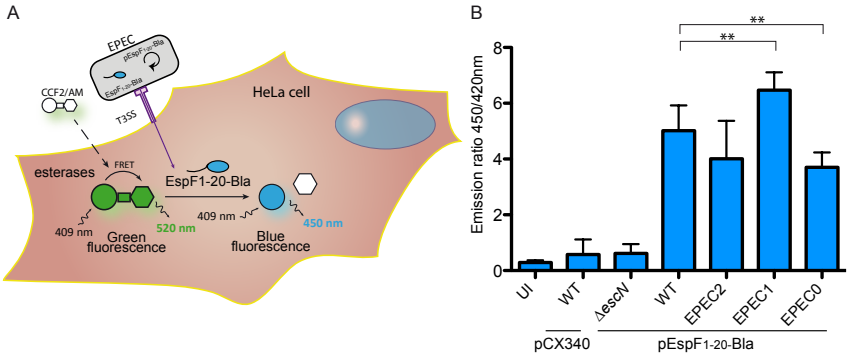

Figure 21. Translocation of β-lactamase (Bla) by EPEC effectors mutant strains. (A) Representation of Bla translocation through the T3SS injectisome of EPEC. $EspF_{1-20}$-Bla fusion is translocated into the mammalian cell. The non-fluorescent Bla substrate (CCF2/AM) is modified by cytoplasmic esterases to the green fluorescent substrate CCF2. CCF2 hydrolysis by Bla results in a shift of green to blue fluorescence. **(B)** HeLa cells were infected 1.5 h and then incubated with CCF2/AM for additional 1 h. Bla activity was quantified measuring the emission ratio of fluorescence at 450/520 nm in HeLa cells infected with the indicated bacterial strains. Results are the mean of three independent experiments with standard deviation (SD). One way ANOVA Tukey's Multiple Comparison Test. **p<0.01

the signal for T3 secretion. HeLa cells were infected with these strains then were incubated with the CCF2/AM Bla substrate. This substrate goes into the cell in a passive mechanism and became fluorescent (green) by the action of eukaryotic esterases, translocation of Bla into the cytoplasm of the HeLa cells lead to the hydrolysis of CCF2 changing its fluorescence emission from 520 nm (green) to 450 nm (Blue), which can be quantified using a fluorimeter. The emission ratio between 450 nm and 520 nm indicated the level of Bla translocation. We found that all strains, except the negative control Δ*escN*, translocate $EspF_{1\text{-}20}$-Bla efficiently into HeLa cells, although small differences in level of translocation were observed. For instance, EPEC1 strain, which is devoid of *espZ*, appears to translocate more Bla than EPECwt, EPEC2 and EPEC0 strains. Conversely, EPEC0, which lacks Tir and intimate adhesion, translocated less Bla than the other strains.

4. Deletion of myosin-interacting domain of EspB in the effector mutant EPEC strains

The main function of EspB is the formation of the translocation pore in the plasma membrane of the host cell for translocation of effectors (Taylor, O'Connell et al. 1998). It has been reported that EspB binds to myosins, which are a superfamily of proteins that interacts with actin filaments and mediate essential cellular processes, like microvilli formation and phagocytosis. EspB binding to myosins mediates the microvilli effacement and phagocytosis inhibition induced by this protein. Thus, deletion of the myosin-interacting domain (mid) of EspB disrupts the microvillus effacement and the inhibition of phagocytosis. It has been reported that deletion of the mid of EspB does not affect the T3SS function and the induction of actin polymerization in the infected host cell (Iizumi, Sagara et al. 2007). With the aim of eliminating the effector function of EspB in the effector-less EPEC strain, we did an in frame deletion of the mid of EspB, encoded between amino acids 159-218 (Iizumi, Sagara et al. 2007). After generation of a Δmid mutant allele of *espB*, we generated strains EPEC0Δmid and EPEC2Δmid. EPEC0Δmid is the effector-less EPEC strain, whereas deletion was also done in EPEC2, because it still induces actin polymerization into HeLa cells, allowing us to test whether EspBΔmid affects Tir translocation. We infected HeLa cells with EPEC2, EPEC2Δmid, EPEC0 and EPEC0Δmid (Figure 22) and observed actin polymerization beneath the adherent bacteria in EPEC2 and EPEC2Δmid. EPEC0 and EPEC0Δmid bound to the HeLa cells and, as expected, they did not induce actin polymerization. Thus deletion of the mid of *espB* does not abrogate the ability of EPEC2 strain to translocate Tir. However we observed fewer bacteria adhered to HeLa cells infected with EPEC2Δmid and also smaller microcolonies. We quantified protein translocation by EPEC2,

EPEC2Δmid, EPEC0 and EPEC0Δmid using Bla translocation assay. We found that Bla translocation was significant lower in EPEC2Δmid and EPEC0Δmid compared to their parental strains (Figure 23).

Hence, although EspBΔmid deletion does not completely disrupt T3SS-dependent translocation, it severely reduces the amount of protein translocation. Therefore,

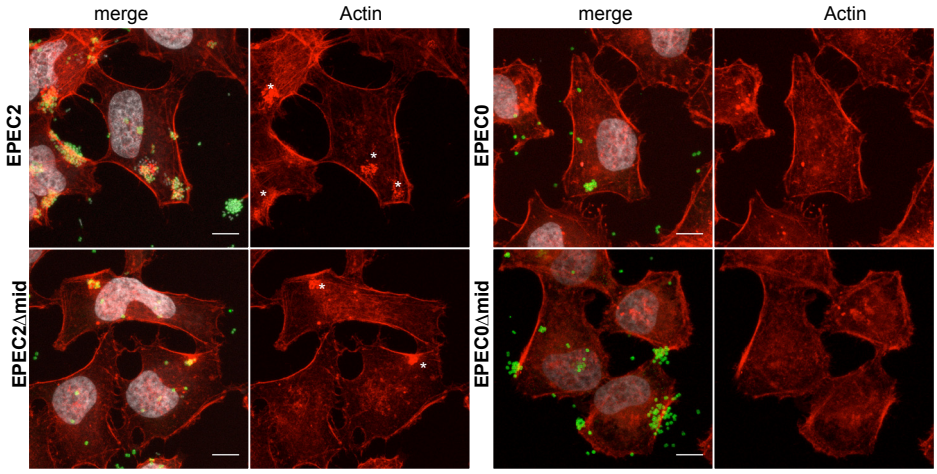

Figure 22. Infection of HeLa cells by EPEC effector mutants expressing EspBΔmid. Immunofluorescence confocal microscopy of Hela cells infected 1.5 h using a MOI 200:1 with EPEC2, EPEC2Δmid, EPEC0 and EPEC0Δmid. EPEC is labeled with rabbit polyclonal anti-intimin-280 serum (green), actin is labeled with TRITC phalloidin (red) and cell nuclei are labeled with DAPI (gray). Actin polymerization is indicated with white asterisks. Scale bar 10 μm.

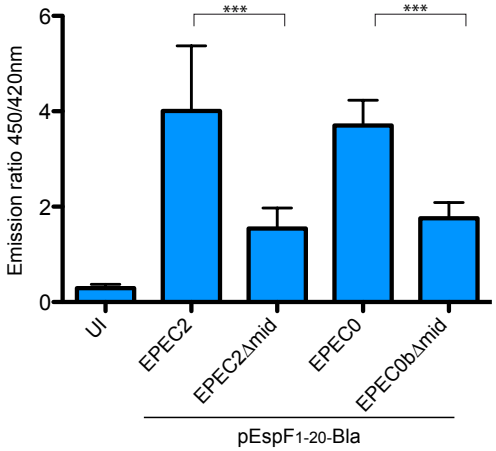

Figure 23. Translocation of Bla into HeLa cells by EPEC effector mutant EspBΔmid. HeLa cells were infected 1.5 h and then incubated with CCF2/AM for additional 1 h. Bla activity was quantified measuring the emission ratio of fluorescence at 450/520 nm in HeLa cells infected with the indicated bacterial strains. Results are the mean of three independent experiments with standard deviation (SD). One way ANOVA Tukey's Multiple Comparison Test. ***p<0.001

to maintain an efficient translocation of proteins the EPEC effector mutant strains required the presence of the mid region of EspB.

5. EspZ downregulates cytotoxicity and Tir translocation levels in HeLa cells infected with effector mutant EPEC strains

EPEC translocates effector proteins to subvert host cellular functions in a way that suits its infection. Because an excess in effector translocation leads to severe cytotoxicity, EPEC employs mechanisms that regulate the intracellular concentration of effectors. EspZ has been reported to downregulate the translocation of EPEC effectors during infection. An EPECΔ*espZ* has higher cytotoxicity during infection of HeLa cells, inducing high level of lactate dehydrogenase (LDH) release compared to EPECwt strain (Berger, Crepin et al. 2012). We wanted to evaluate the role of EspZ in the EPEC effector mutant strains during infection of HeLa cells.

5.1 LDH-release in HeLa cells infected with effector mutant EPEC strains

We infected HeLa cells with EPECwt, EPEC2, EPEC1, EPEC0, and with the mutant EPECΔ*espZ*, which was previously constructed with Δ*espZ-2* allele (see Figure 13). To measure cytotoxicity we used the LDH-release as an indicative of damage in the cell plasma membrane. As expected, EPECΔ*espZ* induced a significant release of LDH compared to uninfected cells or cells infected with EPECwt, which induce low LDH-release (Berger, Crepin et al. 2012). EPEC2 and EPEC0 show low LDH-release

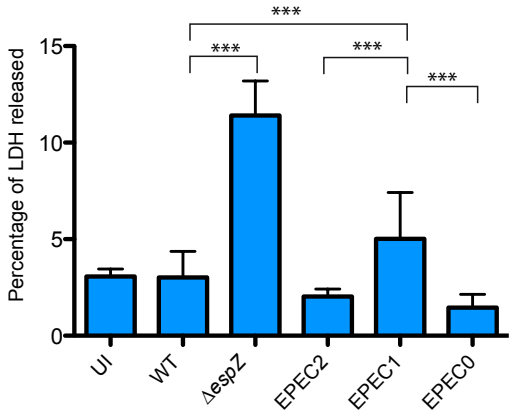

Figure 24. Host cells LDH-release after infection with EPEC effector mutant strains. Percentage of LDL-release compare to the total LDH-release of cells lysed with triton. HeLa Cells were infected for 3 h with EPECwt, EPECΔ*espZ*, EPEC2, EPEC1 and EPEC0. Uninfected (UI) cell were used as a control. Cytotoxicity was measured quantifying the level on LDH release into the culture supernatants in the last 1.5 h of the infection. Results are the mean of three independent experiments with standard deviation (SD). One way ANOVA Tukey´s Multiple Comparison Test. ***p<0.001

similar to the levels released by EPECwt. In contrast EPEC1 shows a significant increase in LDH-release compared to EPECwt, EPEC2 and EPEC0 (Figure 24).

These data suggest that the lack of *espZ* in EPEC1 could deregulate protein translocation by the injectisome and leads to a higher level of translocation of the only remaining effector present in EPEC1, (i.e. Tir). Therefore, the absence of EspZ in EPEC1 may increase the cytotoxicity in HeLa cells due to an excessive translocation of Tir.

5.2 Levels of Tir translocated into HeLa cells infected with effector mutant EPEC strains

We measured the amount Tir translocated by our strains during infection of HeLa cells. For this purpose we performed 3 h infection of HeLa cells with EPECwt, EPECΔ*escN*, EPEC0, EPEC1 and EPEC2. We prepared cellular lysates of HeLa cells and analyzed Tir level by Western blot (Figure 25). We found Tir in HeLa cells infected with EPECwt, EPEC2 and EPEC1 but not in cells infected with EPECΔ*escN* and EPEC0 strains. Interestingly, the translocated levels of Tir were much higher in EPEC1 than in EPEC2 and EPECwt strains demonstrating that absence of EspZ increase the level of translocated Tir. In addition, the levels of Tir in cells infected

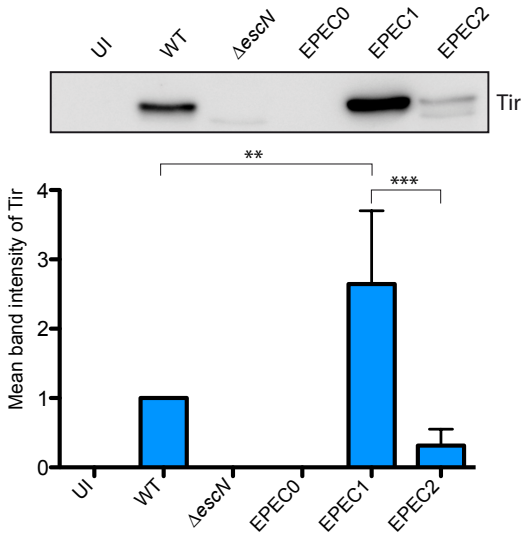

Figure 25. Levels of Tir translocated in HeLa cells by effectors mutant EPEC strains. Western blot of cell lysates with anti-Tir mAb . HeLa infected for 3 h with EPECwt, EPECΔescN, EPEC0, EPEC1 and EPEC2. Uninfected (UI) HeLa cells were used as control. Quantification of Tir band intensity in the indicated strains. Results are the mean of three independent experiments with standard deviation (SD). 1 way ANOVA Tukey´s Multiple Comparison Test. **p<0.01, ***p<0.001.

with EPEC2 were lower than those found in cells infected with EPECwt. Small differences in protein translocation levels found between EPECwt and EPEC2 strains (Figure 21) may explain, at least partially, the difference in the levels of Tir in infected HeLa cells. Nonetheless, this result may also suggest that additional effector(s) that are absent in EPEC2 could be necessary to stabilize Tir in the infected cell.

6. Translocation of individual LEE and non-LEE effectors by the effectors mutant EPEC strains

We wanted to evaluate the translocation of individual effectors in our effector mutant strains. In order to maintain physiological expression levels, we reintegrated a single copy of the effector gene of interest in its native location in the chromosome of EPEC2, EPEC1 and EPEC0, using suicide vectors with homology regions flanking the corresponding effector gene (Figure 8). Hence, similarly to gene deletion strategy, we followed a marker-less strategy for gene integration that preserves genome context and native regulatory elements (i.e., promoters, RBS, terminators) of effector genes. We followed this strategy to integrate effectors *espH* and *map* in the LEE and *nleC* in PP4.

6.1. Translocation of EspH induces focal adhesions disassembly and cell rounding

EspH is one of the LEE-encoded effectors, that modulate host actin cytoskeleton (Tu, Nisan et al. 2003). EspH induces focal adhesions (FAs) disassembly (Wong, Clements et al. 2012). FAs are dynamic complexes of proteins that are localized at the site between the cell and the extracellular matrix (ECM) (Sastry and Burridge 2000). Vinculin is a protein associated to these protein complexes (Humphries, Wang et al. 2007). We evaluated whether the translocation of EspH into HeLa cells by effector mutant EPEC strains complemented with *espH* in the chromosome induced FAs disassembly. Cultured HeLa cells were infected with EPECwt and with EPEC2, EPEC1 and EPEC0 with and without *espH* integrated and stained to visualize actin and vinculin by immunofluorescence microscopy (Figure 26).

This experiment showed that EPECwt, EPEC2+*espH* and EPEC1+*espH* induced disruption of the FAs in the infected cells, whereas HeLa cells infected with strain lacking *espH* did not induce FAs disassembly, showing a vinculin staining pattern similar to UI control cells (Figure 26). Under these conditions of infection EPEC0+*espH* did not induce disassembly of FAs likely due the weaker cell attachment

of this strain. A dramatic cell rounding phenotype also evidences the disruption of the host actin cytoskeleton by EspH (Dong, Liu et al. 2010, Wong, Clements et al. 2012). We quantified HeLa cell rounding by the individual translocation of EspH after 2 h and 3 h of infection with EPECwt and effector mutant strains (Figure 27).

After 2 h of infection, EPECwt induces a clear cell rounding phenotype in 36% of

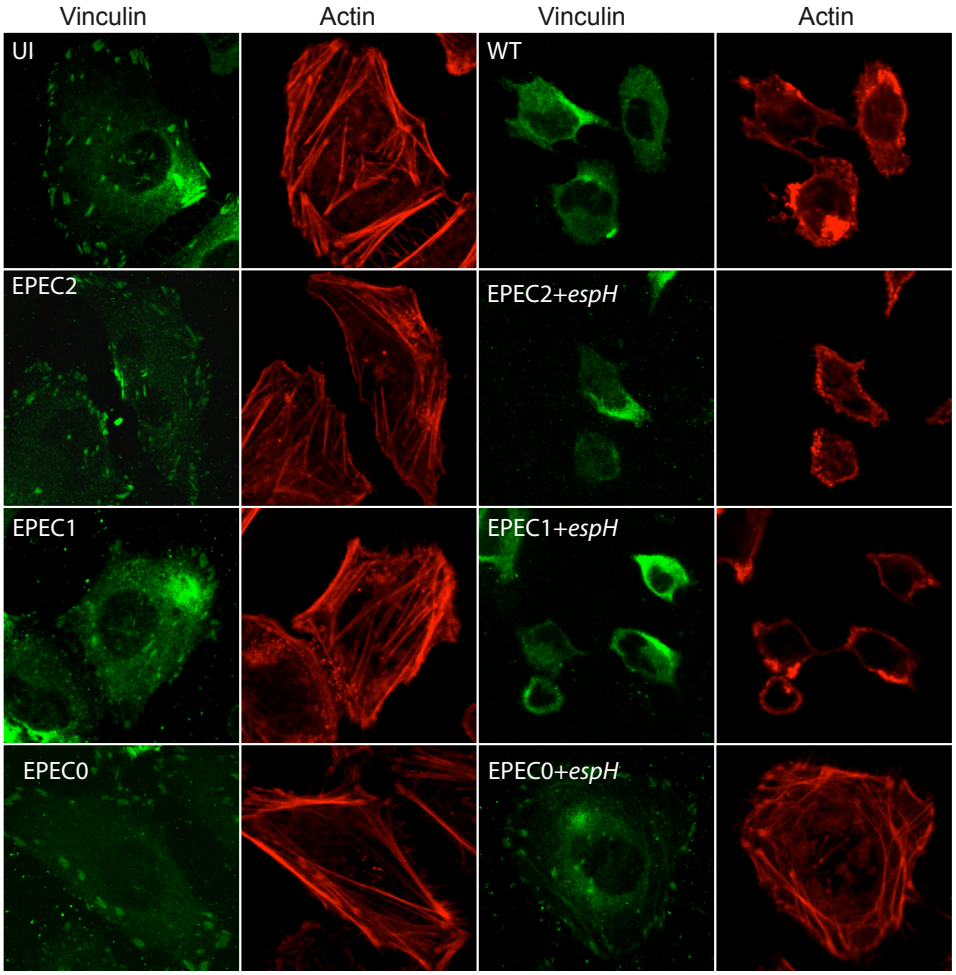

Figure 26. Individual translocation of EspH by effectors mutant strains induces FAs disassembly. Immunofluorescence confocal microscopy of HeLa cells infected 1 h plus 1 h with gentamicin treatment (MOI 100:1), with EPEC2, EPEC1 and EPEC0 and isogenic strains with espH integrated in the chromosome. Control of uninfected (UI) and cells infected with EPECwt are shown. Vinculin was detected with anti-vinculin antibody (green) and actin was labeled with TRITC phalloidin (red).

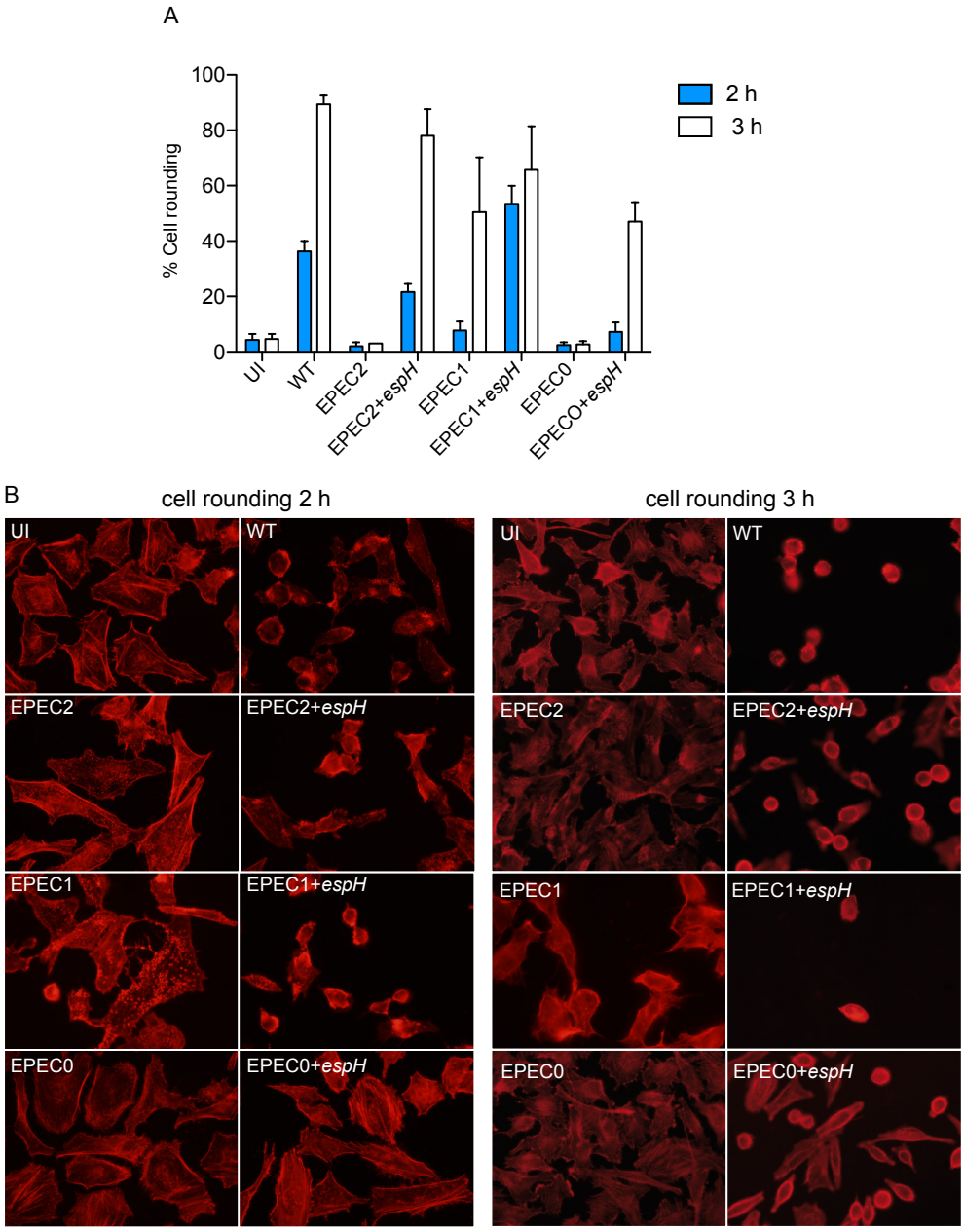

Figure 27. Individual translocation of EspH by effector mutant strains induces cell rounding. (A) Percentage of cell rounding on HeLa cells infected during 2 h (1 h with gentamicin treatment) (MOI 100:1) and 3 h (MOI 200:1), with EPEC2, EPEC1 and EPEC0 with espH integrated in the chromosome and with the isogenic control strains without *espH*. Control of uninfected (UI) and cells infected with EPECwt are shown. One hundred cells were counted in triplicate. Results are the mean of three independent experiments with standard deviation (SD). **(B)** Immunofluorescence microscopy of HeLa cells infected 2 h (1 h with gentamicin) and 3 h with the indicated bacterial strains. Actin was labeled with TRITC phalloidin (red).

the cells whereas EPEC2 and EPEC0 strains do not induce cell rounding. EPEC1 induces cell rounding in 7% of the cells and this small increase in cell rounding compared to EPEC2 and EPEC0 is likely caused by the cytopathic effect of an excess of translocated Tir. The strains EPEC2+*espH* and EPEC1+*espH* induce higher levels of cell rounding, in 21% and 53% of the cells, respectively. The high level of cell rounding caused by EPEC1+*espH* could be due to higher levels of Tir and EspH translocation. Under these infection conditions (2 h) EPEC0+*espH* induced low levels of cells rounding (i.e. 7% of the cells). Longer infection of 3 h increased the cell rounding phenotype in all strains with *espH*, including EPE0+*espH* (Figure 27A). Importantly, HeLa cells infected with EPEC2 and EPEC0 remained well attached after 3 h of infection, with similar appearance to uninfected control cells (Figure 27B). After 3h of infection, EPEC1 strains showed severe cytopathic effect on HeLa cells and also induced high level of cell detachment (Figure 27B). Together, these results demonstrate that effector mutant EPEC strains were able to translocate functional EspH from chromosomal expression.

6.2. Translocation of Map induces filopodia

The mitochondrial associate protein (Map) is a multifactorial effector that induces filopodia in the cell surface at early time of infection and mitochondrial dysfunction at late time of infection (Papatheodorou, Domanska et al. 2006, Berger, Crepin et al. 2009). Filopodia are thin, actin-rich plasma membrane protrusions like spikes associated with the infecting microcolonies in the cell surface (Mattila and Lappalainen 2008, Berger, Crepin et al. 2009). Cultured fibroblast Swiss 3T3 cells were infected for a short time (10 min) with EPEC2, EPEC1 and EPEC0 strains and isogenic strains with a single copy of *map* in its endogenous gene locus. In this experiment we centrifuged the plate with the cells and bacteria at the beginning of the infection to synchronize infection and increase early cell contacts even in EPEC0 strains. Actin staining of infected cells for immunofluorescence microscopy revealed the induction of filopodia by the effector mutant EPEC strains carrying *map* (Figure 28).

This indicates that individual translocation of Map, from chromosomal expression, induces filopodia formation similar to the reported filopodia associated with EPECwt microcolonies (Jepson, Pellegrin et al. 2003, Huang, Sutton et al. 2009).

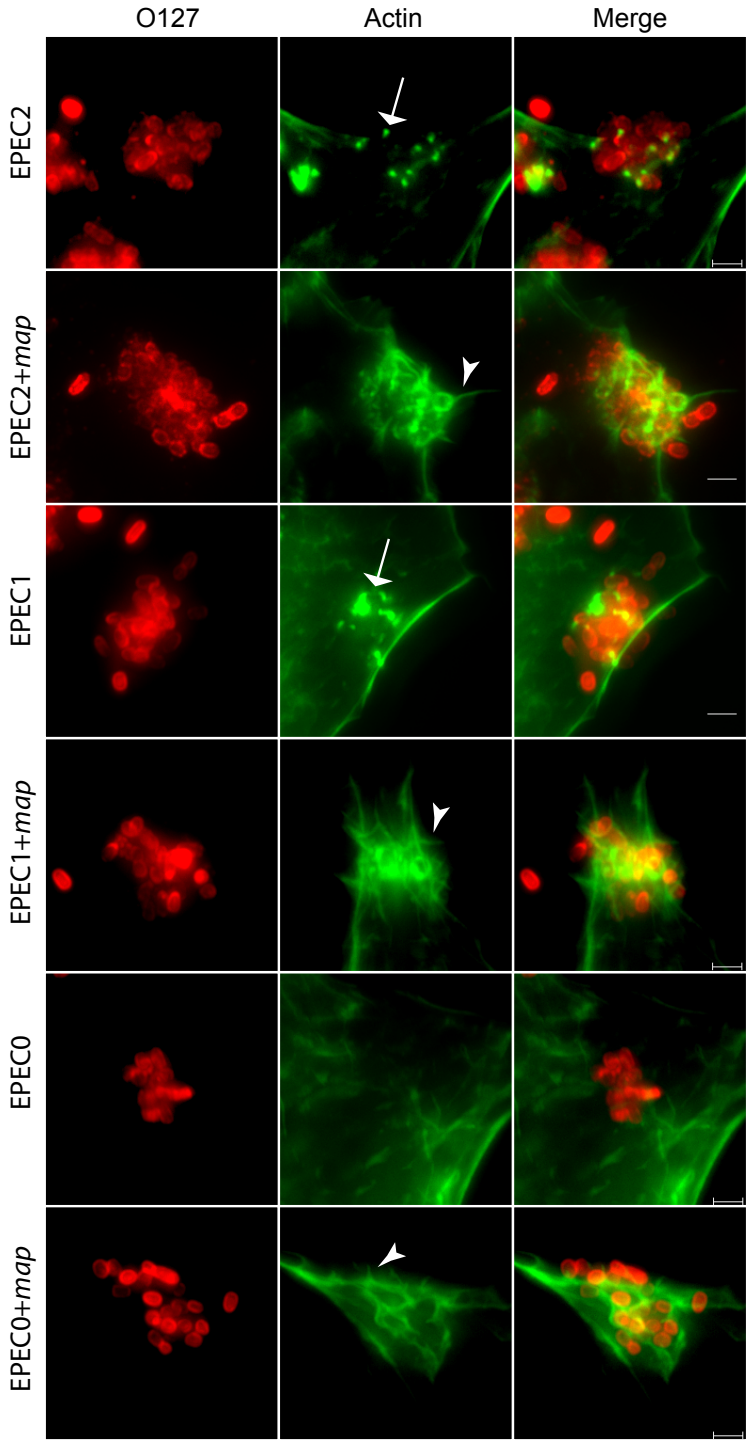

Figure 28. Individual Translocation of Map by effector mutant strains induces filopodia. Immuno fluorescence microscopy of 3T3 cells infected for 10 min with EPEC2, EPEC1 and EPEC0 and isogenic strains with map integrated in the chromosome. EPEC was detected with rabbit polyclonal anti 0:127 (red) and actin was stained with Orego-green phalloidin (green). Filopodia like spikes (arrowheads). Actin-polymerization (arrows). Scale bar 2 μm.

6.3. Translocation of NleC degrades p65 of the NF-κB complex

During EPEC infection different effectors are used by the pathogen to block the Nf-κB proinflammatory pathway (Newton, Pearson et al. 2010, Li, Zhang et al. 2013). NleC degrades p65 component of NF-κB to decrease the cytoplasmic levels, disrupting p65 nuclear translocation and cytokine gene expression (Pearson, Riedmaier et al. 2011). It has been postulated that NleC preferentially targets free p65 released from the IκB complex, although it can also degrade bound p65 (Pearson, Riedmaier et al. 2011). The *nleC* gene was reintegrated in its native locus of the chromosome of EPEC2, EPEC1 and EPEC0. Next, we infected HeLa cells (Figure 29) for 1 h, and then cells were washed and incubated for additional 3

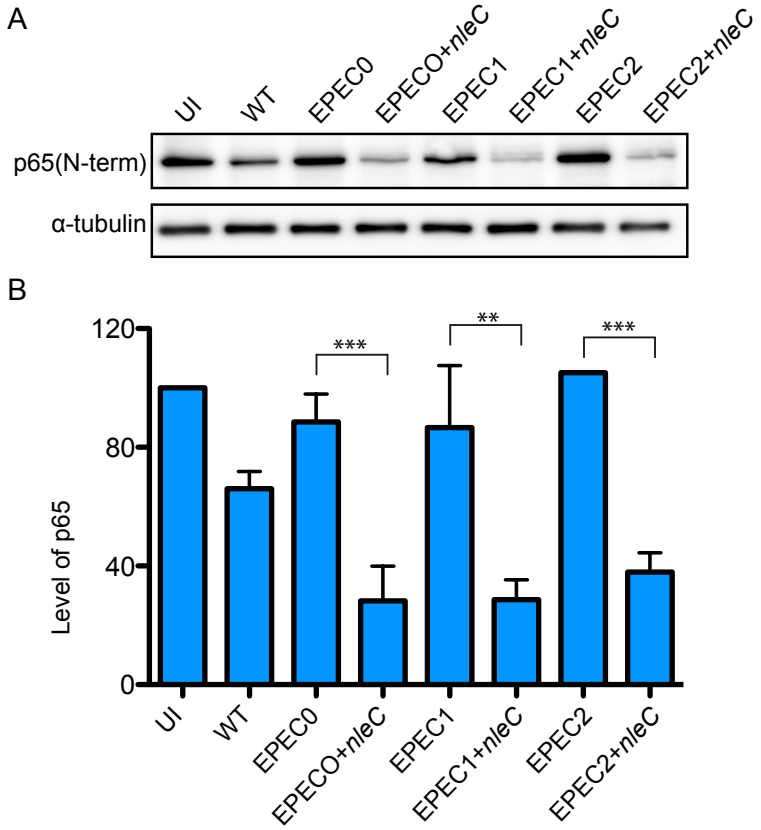

Figure 29. Translocation of NleC by EPEC effectors mutant strains induces p65 degradation. (A) Western blot displaying p65 of HeLa cells infected for 4h (3 h with gentamicin) with EPEC2, EPEC1 and EPEC0 with *nleC* integrated in the chromosome and with the isogenic parental strains without nleC and EPECwt. Uninfected (UI) cell used as control. Cell lysates were analyzed by Western blots and p65 was detected with rabbit polyclonal anti NF-κB p-65. α-tubulin was used as a loading control and it was detected with mouse monoclonal anti-alpha tubulin. **(B)** Quantification of p65 in Hela cells infected with the indicated strains. Protein loading was normalized with α-tubulin. Results are the mean of three independent experiments with standard deviation (SD). One way ANOVA Tukey´s Multiple Comparison Test. **P <0.01, ***P <0.001.

h with gentamicin in DMEM. Western blots of the cell lysates revealed that p65 was proteolysed in cell infected with the effectors mutant strains carrying *nleC*. Proteolysis of p65 in effectors mutant strains carrying *nleC* was higher than that induced by the EPECwt strains, likely caused by the presence of other effector in the wild type strains which prevent IκB degradation.

7. Evaluation of A/E formation in human biopsies by the effector mutant EPEC strains

The previous results with HeLa cells indicated that all effector mutant strains carrying Tir are able to induce reorganization of the actin filaments in pedestal-like structures, underneath the attached bacteria. This includes EPEC1, which carries Tir in the absence of all other effectors. Hence, we wanted to evaluate whether these EPEC effectors mutant strains were also able to induce A/E lesion formation in human intestinal biopsies, a model closer to natural human infection.

7.1. EPEC2, EPEC1 and EPEC0 do not induce A/E lesion in human intestinal biopsies

We infected human intestinal biopsies with the effector mutant EPEC strains. After 8 h of infection, biopsies samples were thoroughly washed, fixed and analyzed by scanning electron microscopy (SEM) to determine the presence of A/E lesions. As previously described (Introduction), A/E lesions are characterized by microcolony formation, brush border microvilli disruption at the site of the infection, and elongation of the microvilli in the periphery of the microcolony (Figure 30). To avoid false positive results we carefully inspected the mucosa surface, avoiding damaged areas that could induce no specific adhesion of the bacteria to the enterocytes. With this analysis we found that EPECwt induces A/E lesion in 76% of the infected biopsies, whereas EPEC0 control did not show A/E lesion formation in none of the infected biopsies (Table 8). Interestingly EPEC2 and EPEC1 strains were no able to induce the A/E lesion in human intestinal biopsies, despite inducing actin polymerization in HeLa cells. These results indicate that other effectors besides Tir and EspZ are essential to induce the A/E lesion formation.

Table 8. Human biopsies infected by EPECwt and EPEC effector mutant strains

Strain	Effectors genes remaining	Number of positive biopsies with A/E lesion Positive/Total (%)
WT	all	13/ 17 (76)
EPEC2	*espZ* and *tir*	0/6 (0)
EPEC1	*tir*	0/6 (0)
EPEC0	none	0/5 (0)
EPEC9	*espZ+tir*+IE2+IE5+IE6+PP2+PP3+PP4+PP6*	5/6 (83)
EPEC2-LEE+	*espZ+tir+map+espH+espF+espG*	0/5 (0)

* Encoded effectors in IEs and PPs

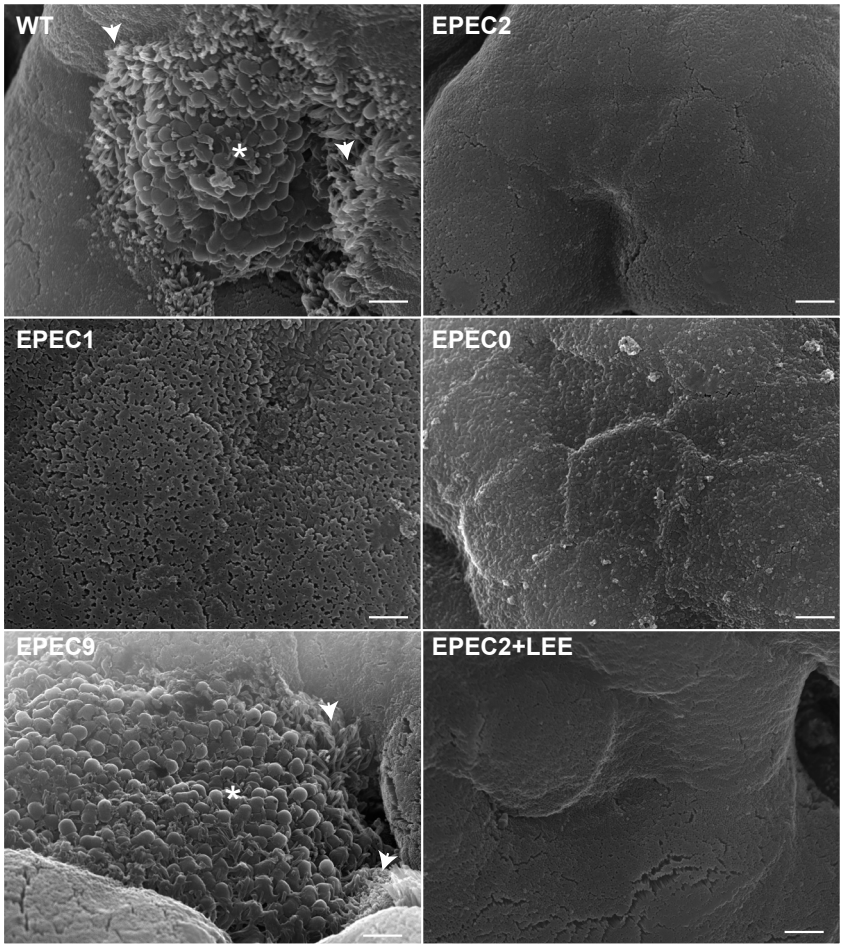

Figure 30. Infection of human duodenal biopsies infected with EPECwt and effectors mutant strains: EPEC2, EPEC1, EPEC0, EPEC9 and EPEC2-LEE+ analyzed by SEM. EPECwt and EPEC9 induce the characteristic A/E lesion in the intestinal mucosa surface: with bacterial microcolony (asterisk) and microvilli elongation in the periphery of the microcolony (arrowheads). The biopsies infected with EPEC2, EPEC1, EPEC0 and EPEC2-LEE+ lack of adherent bacteria and A/E lesions, showing normal microvilli on enterocyte surface of human biopsies. Scale bar 2 µm

7.2. Non-LEE effectors are essential for the induction of A/E lesion in human biopsies

To characterize whether LEE or non-LEE effector(s) were needed to induce A/E lesion in human intestinal biopsies assay we performed infections with the effectors mutant strains EPEC9 and EPEC2-LEE$^+$ (Figure 30). EPEC9 strain maintains two LEE effectors (EspZ and Tir) and the whole repertoire of non-LEE effectors found in EPECwt. EPEC2-LEE$^+$ is a strain derivative of EPEC2 (*espZ*+ *tir*+), in which we introduced the deleted LEE effectors (*espG*, *map*, *espF*, *espH*) to reconstitute a wt LEE, but that is devoid of all the repertoire of non-LEE effectors found in EPECwt. Infection of human biopsies revealed that EPEC2-LEE$^+$ strain is unable to induce efficient A/E lesion formation whereas EPEC9 strain induces A/E lesion formation at levels similar to EPECwt (Table 8). In all experiments we infected human biopsies with EPECwt to have positive controls of A/E lesion, which explains the higher number of biopsies infected with this strain. Hence, these results indicate that non-LEE effectors are essential to induce the of A/E lesion formation in human intestinal biopsies.

To characterize the non-LEE effector(s) participating in the A/E lesion formation, we analyzed infection of human biopsies with strains having sequential deletions of the clusters of effectors genes present in integrative element: IE5, IE6 and IE2 (Table 9). While deletion of IE5 (EPEC8) did not have a significant effect on A/E lesion formation, the percentage of A/E positive biopsies decreased to 54% with deletion of IE6 (EPEC7) and to 23% by the deletion of IE2 (EPEC6).

Table 9. Human biopsies infected by EPEC effector mutant strains

Strain	Effectors genes remaining	Number of positive biopsies with A/E lesion Positive/Total (%)
EPEC9	espZ+tir+IE2+IE5+IE6+PP2+PP3+PP4+ PP6*	9/11(82%)
EPEC8	*espZ+tir*+IE2+IE6+PP2+PP3+PP4+PP6*	10/14(71)
EPEC7	*espZ+tir*+IE2+PP2+PP3+PP4+PP6*	7/13(54)
EPEC6	*espZ+tir*+PP2+PP3+PP4+PP6*	3/13(23)
EPEC7Δ*nleE2*	*espZ+tir*+ *lifA*-like+PP2+PP3+PP4+PP6*	7/11(64)
EPEC7Δ*efa1*/ *lifA*-like	*espZ+tir*+ *nleE2*+PP2+PP3+PP4+PP6*	4/12(33)

* Encoded effectors in IEs and PPs

The dramatic reduction in the efficiency of A/E lesion formation in EPEC7 and EPEC6 strains indicates that deletion of IE6 and IE2 notably reduces the capacity of EPEC to induce the A/E lesion formation in human biopsies. Interestingly, EPEC6 strain having only PPs encoded-effectors was still capable of inducing A/E lesion formation in 23% of samples analyzed. This indicates an additive role of the non-LEE encoded effectors. The IE6 and IE2 have almost identical clusters of effectors genes. IE6 carries one copy of *espL*, *nleB1*, *nleE1* and *efa1/lifA* whereas IE2 carries a pseudogene copy of *espL** and *nleB**, one copy of *nleE2* and one copy *efa1/lifA*-like, a gene homolog of *efa1/lifA* (Figure 31A). This suggests that the copies of *nleE* and/or *efa1/lifA* homologs could play a major role in A/E lesion formation. To investigate this possibility we generated individual deletions of *efa1/lifA*-like and *nleE2* of the IE2 in EPEC7, which already has a deletion in IE5 and IE6 (Figure 31A). Human biopsies infected with EPEC7Δ*nleE2* strain (carrying a single functional copy of *efa1/lifA*-like) induced A/E lesion in 64% of the infected biopsies, similar to EPEC7. On the contrary, deletion of *efa1/lifA*-like in EPEC7 reduced the A/E lesion formation to 33% of the infected biopsies similar to EPEC6 (Table 9). We confirmed by RT-PCR that deletion of *nleE2* or *efa1/lifA*-like have no polar effects on the expression of the remaining genes in the IE2 (Figure 31B).

These results indicate that deletion of *efa1/lifA*-like has a major impact on A/E lesion formation, explaining the low efficiency of A/E lesion formation in EPEC6. Collectively, these results demonstrate the requirement of non-LEE effectors for A/E lesion formation, with an additive role of effectors encoded in integrative elements (IEs) and prophages (PPs), and suggest a major role of *efa1/lifA* proteins in this process.

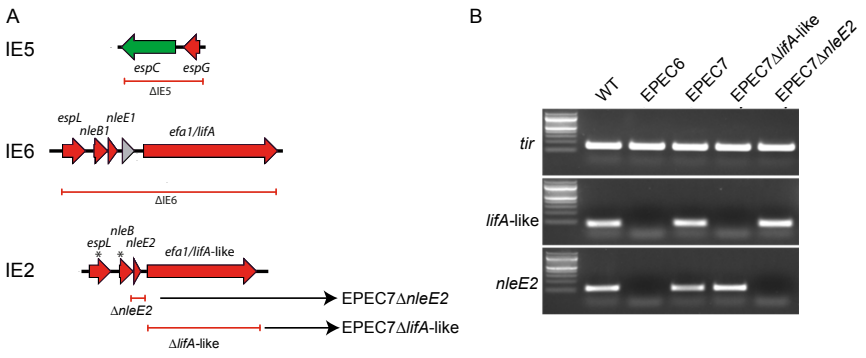

Figure 31. Deletion of nleE2 and efa1/lifA-like in EPEC7. (A) Clusters of IEs encoded-effectors in IE5, IE6 and IE2. Red bars indicated deletion of IE5 and IE6 encoded-effectors and individual deletion (*nleE2* and *lifA*-like) in EPEC7Δ*nleE2* and EPEC7Δ*lifA*-like. (B) Electrophoresis gel of RT-PCR products of expression of *lifA*-like and *nleE2* in effectors mutant strains. EPEC7Δ*lifA*-like has expression of *nleE2* and EPEC2Δ*nleE2* has expression of *lifA*-like. EPEC7 has expression of *lifA*-like and *nleE2*. EPEC6 does not have expression of *lifA*-like and *nleE2*. The expression of *tir* was used as a control for RT-PCR.

DISCUSSION

Generation and characterization of the effector mutant EPEC strains

EPEC as other pathogens uses its T3SS to translocate effectors proteins into the cells to subvert host cellular functions. EPEC was the first *E. coli* pathogen to be associated with human disease and many studies have investigated the role EPEC effectors. In this PhD thesis we generated several effector mutant strains with the aim of further characterizing the role of EPEC T3SS effectors in infection and broaden the knowledge about EPEC colonization of human intestinal mucosal surface. In addition, the attenuation of the effector mutant EPEC strains could be of biotechnological interests, for instance for the development of an EPEC vaccine and for the delivery of therapeutic proteins into enterocytes of human intestine. Since our objective was to make multiple deletions in the genome of EPEC and reintroduce selectively specific effector(s) in the strain, we wanted the use an efficient deletion strategy that would allow us to made gene deletions and integrations in the genome without leaving antibiotic resistance markers or recombination sequences ("scars") in the chromosome of the engineered strain. Several techniques allow genome engineering based in homologous recombination to delete and integrate target genes. These techniques are based on generating a mutant allele *in vitro* and later introduce it in the bacteria to replace the target gene by homologous recombination. Gene modification can be conducted introducing the mutant allele in the bacterium as a linear DNA fragment and expressing the λ Red genes for recombination, or cloned in a suicide plasmid that is integrated and resolved by means of antibiotic resistance gene markers and counter-selection systems (e.g. sucrose/*sacB*) (Datsenko and Wanner 2000, Mizoguchi, Tanaka-Masuda et al. 2007). However, frequently these techniques leaves "scars" sequences after removal of antibiotic markers or the counter-selection system are not efficient in *E. coli*. We chose the markerless gene deletion strategy previously described by Posfai and colleagues (1999), which is based on the integration by a single homologous recombination event of a suicide plasmid carrying the mutant allele and I-*Sce*I restriction sites. This co-integrate is resolved by a second intramolecular recombination event induced by the generation of a double strand break in the chromosome by cleavage of I-*Sce*I restriction enzyme, which can be transiently expressed in the bacteria upon induction.

This methodology had been successfully employed to minimize the genome of *E. coli* K-12 strain (Posfai, Plunkett et al. 2006).

The prototypical EPEC strain E2348/69 has been widely studied using classical genetic and global "omics" approaches providing a good knowledge of the whole repertoire of effectors encoded in the genome of this strain (Iguchi, Thomson et al. 2009, Deng, Yu et al. 2012). Taking advantage of this information and the markerless gene deletion strategy based on I-*Sce*I, we carried out the deletions necessary to build an effector-less EPEC strain (EPEC0) devoid of all known T3SS effectors. We successfully delete all the effectors genes reported in the chromosome of EPEC E2348/69 through 13 deletions, being 326 bp the smallest deletion (*espZ*) and the 18260 bp the largest deletion (cluster of effectors in IE6). All the effector genes deleted were eliminated from the start to the stop codon, maintaining their original transcriptional promoters and terminator signals without leaving any scars in the chromosome. The only exception to this global approach was the deletion of *espZ*, in which deletion of its RBS was necessary to maintain correct expression levels of the downstream genes in the operon. We verified the deletions made in the genome by PCR and by whole genome sequencing comparison of the parental and the effector mutant strain EPEC1, in which Tir effector is still encoded. We sequenced EPEC1 genome because translocation of Tir to HeLa cells allowed us to confirm the functionality of T3SS in this strain.

A central objective of our strategy was to keep a functional T3SS in EPEC. Therefore, we decided to delete first the effector genes localized in the LEE island to evaluate whether any of these deletions could disrupt the correct assembly and functionality of the T3SS. The effector genes *espZ* and *tir* are also localized in the LEE but we left these two genes for the last two deletions because EspZ and Tir effectors are important to control physiological protein translocation levels and intimate bacterial attachment to host cells. It was previously reported that EspZ blocks effector protein translocation from a second wave of EPEC infection (Mills, Baruch et al. 2008, Berger, Crepin et al. 2012). Therefore, we decided to maintain EspZ to have a controlled translocation of the remaining effector proteins during

the construction of the effector-less EPEC strain. Besides, *tir* was the last gene deletion because translocation of Tir is essential for intimate bacterial attachment and it is a helpful indicator of the functionality of the T3SS through its actin-polymerization phenotype upon translocation (Kenny, DeVinney et al. 1997).

We confirmed that the multiple deletions generated in the chromosome of EPEC do not have deleterious effect on the growth of the bacterium, since EPECwt, EPEC2, EPEC1 and EPEC0 strains have the same growth rate, viability and cellular morphology in LB and DMEM media (Figure 16). We also evaluated after each deletion the correct assembly of the T3SS injectisome. The mutant strains were grown under induction conditions and the secretion of EspABD translocator proteins was confirmed by SDS-PAGE (Figure 17). The correct expression level of some structural proteins of the T3SS injectisomes, like EscC, EscJ, EscD and the translocator protein EspB was confirmed by Western blot in all the effector mutant strains (Figure 18). Next, we infected HeLa cells with the EPECwt and the effector mutant EPEC strains to evaluate the functionality of the T3SS. EPEC2 (bearing *espZ* and *tir*) and EPEC1 (bearing only *tir*) were able to induce the actin-pedestal formation underneath the attached bacteria. This result allowed us to conclude that EPEC only needs the effector Tir to induce the actin-pedestal during infection of epithelial cells *in vitro* (Figure 19). As expected, the effector-less EPEC0 strain did not induce the actin polymerization because Tir is essential to start the actin polymerization cascade. We performed protein translocation assays of Bla to quantitate protein translocation in the effector mutant EPEC strains, including EPEC0 strain (Figure 21). All effector mutant strains translocate Bla fused to the N-terminal 20 amino acids of EspF ($EspF_{1-20}$-Bla) into HeLa cells. EPEC2 translocates Bla at slightly reduced levels compared to EPECwt, but the difference was not statistically significant, suggesting that EspZ and Tir effectors alone are sufficient to control protein translocation at levels close to those found in EPECwt. On the contrary, EPEC1 translocates higher amounts of Bla than the EPECwt, likely due to the absence of the EspZ effector. It has been proposed that EspZ, which inserts in the host plasma membrane, controls protein translocation by interacting with an unknown bacterial protein via an extracellular loop,

which induces a conformational change at the N-terminus that interacts with EspD translocator blocking protein translocation (Berger, Crepin et al. 2012). EPEC0 reduces the level of Bla translocation into the cells likely because in the absence of intimate bacterial adhesion protein translocation is less efficient. This assay also allowed us to confirm that all effector mutant EPEC strains, including EPEC0, have a functional T3SS for protein translocation.

The T3-secreted protein EspB is essential for the formation of the protein-translocation pore, but is also targeted to the cytoplasm of the infected cells and has effector function (Taylor, O'Connell et al. 1998, Luo and Donnenberg 2011). EspB within HeLa cells induces redistribution of actin altering the cell shape (Taylor, Luther et al. 1999). EspB interacts with myosin within the cell through a central domain called mid. Myosin proteins interact with actin filaments mediating essential cellular processes like microvillus formation and phagocytosis. EspB interaction with myosins is responsible of the microvilli disruption and phagocytosis inhibition. It was reported that an EPEC mutant with deletion of the mid domain of EspB maintains T3SS functionality but could not induce microvilli effacing or suppress phagocytosis (Iizumi, Sagara et al. 2007). In an attempt to delete all effector functions in the effector-less strain, we conducted deletion of the EspB mid domain in EPEC0 to generate EPEC0Δmid strain. We did the same deletion in EPEC2 generating EPEC2Δmid to test translocation of Tir in this strain. Infections of HeLa cells with these mutants (Figure 22) allowed us to confirm that EPEC2Δmid was able to translocate Tir, but the size of bacterial microcolonies was smaller compared with the parental strain EPEC2 indicating less adhesion of the bacteria to the cells. We evaluated the functionality of the T3SS performing Bla translocation assays to compare EPEC2, EPEC0 and their *espB*Δmid mutant strains (Figure 23), which showed that deletion of the mid domain of EspB strongly reduces protein translocation. Since our main objective was to maintain physiological levels of protein translocation in the effector mutant EPEC strains, we did not use strains with the *espB*Δmid deletion in our subsequent experiments.

It has been reported that EPEC Δ*espZ* strain is highly cytotoxic, likely due to deregulated translocation of protein effectors (Berger, Crepin et al. 2012). We observed that EPEC1 strain, which is devoid of EspZ, has an increased protein translocation of Bla. Towards further characterize the role of EspZ effector we conducted a cytotoxicity assay to measure the level of LDH released from HeLa cells after infection with the effector mutant EPEC strains. We found not significant difference of LDH-release between uninfected cells and cells infected with EPECwt, EPEC2, and EPEC0. Importantly, we found that EPEC1 is highly cytotoxic and induces a strong LDH-release (Figure 24). Tir is the only remaining effector in EPEC1. Hence, we measured the level of Tir in HeLa cells after infection with the effector mutant EPEC strains. Tir was found translocated into HeLa cells by EPECwt, EPEC2 and EPEC1 and, as expected, was absent in cells infected with control strains (EPEC0 and EPECΔ*escN*). We found that EPEC1 injects significantly higher level of Tir into HeLa cells (Figure 25). This suggests that the cytotoxicity of EPEC1 could be due for this higher amount of Tir translocated into the cell. A possibility is that this uncontrolled translocation of Tir increases the number of Tir molecules inserted in the cell membrane allowing Intimin-Tir interaction and inducing an increase number of intimate attached bacteria to the cell. This "superinfection" should increase the number of T3SS injectisomes in contact with the cell and probably this high number of translocation pores is responsible for the cytotoxic effect of EPEC1, although direct cytotoxic effects due to high levels of Tir in the cytosol of the infected cell cannot be excluded. Interestingly, these experiments also reveal low levels of Tir in HeLa cells infected with EPEC2. The difference in Tir levels between cells infected with EPEC2 and EPEC1 emphasizes the role of EspZ to control protein translocation. Since EPEC2 did not show a strong reduction of protein translocation in the Bla assay, these results suggest that EPEC2 might be devoid of an effector that could potentially stabilize Tir in the infected cell. It is worth noting that the reduced amount of Tir found in HeLa cells infected by EPEC2 is sufficient to induce actin-pedestals that cannot be distinguished from those of the wild type strain (Figure 19). Collectively, these results further demonstrate that EspZ is able to downregulate protein translocation levels reducing the cytotoxicity associated to high levels of effectors and T3SS.

Translocation of EspH, Map and NleC using effector mutant EPEC strains

To understand bacterial virulence it is important to characterize bacterial effectors, their host targets and their mechanism of action. Several approaches have been used to study bacterial effector function in the host. The ectopic expression of an effector into eukaryotic cells by transfection is useful to get information about the effector phenotypes (Clements, Smollett et al. 2011, Wong, Clements et al. 2012). A different approach is to study the phenotype of mutant strains and to evaluate whether the strain recover its wild type phenotype by *trans*-complementation (Berger, Crepin et al. 2012, Wong, Clements et al. 2012). We wanted to test whether translocation of individual effectors from chromosomal single-copy reintegration in the effector mutant strains could reproduce phenotypes previously reported with mutant strains and transfection experiments. We chose EspH, Map and NleC effectors for these experiments since they target different cellular activities and were located in distinct loci in the LEE and outside the LEE (Berger, Crepin et al. 2009, Dong, Liu et al. 2010, Pearson, Riedmaier et al. 2011). Therefore, we reintegrated *espH* and *map* in the LEE and *nleC* in the PP4 in their original site and under native endogenous transcriptional control signals in EPEC2, EPEC1 and EPEC0.

The Rho GTPases are important regulators of actin cytoskeleton and participate in important cellular processes as migration, adhesion, morphogenesis and phagocytosis (Schmidt and Hall 2002). The activation and inactivation of the Rho GTPases is regulated, among other mechanisms, by guanine nucleotide exchange factors (GEFs), which promote dissociation of GDP and subsequent binding of GTP (Wong, Clements et al. 2012). EspH effector directly binds RhoGEF, competing with Rho for binding to RhoGEF and preventing Rho activation. The actin cytoskeleton of the cell is link to the extracellular matrix through FAs, it has ben reported that EspH induces FAs disassembly (Wong, Clements et al. 2012). Ectopic expression of EspH and trans-complementation experiments from overexpressing plasmids have shown that EspH alters the cellular shape inducing cell rounding and detachment (Dong, Liu et al. 2010). We did immunofluorescence microscopy

of HeLa cells infected with the effector mutant EPEC strains with and without *espH* integrated in the chromosome (Figure 26 and 27). All the strains with *espH* are able to induce cell rounding and FAs disassembly. Also EPEC0+*espH* can induce cell rounding but in this case EPEC0 needs more time of infection to rich the translocated protein level necessary to induce the phenotype, because EPEC0 is devoid of Tir to help with the intimate attachment, which favors the translocation(Battle, Brady et al. 2014). With the effector mutant strains we demonstrated that the individual translocation of EspH was sufficient to induce FAs disassembly and cell rounding from native expression levels without the need the other effector(s).

Map is a multifunctional effector protein that induces a transient filopodia formation at the site of bacterial attachment and also induces mitochondria dysfunction. These properties of Map are carried out in different domain of the protein, as mutation in the WxxxE motif of Map involved in filopodia formation abolished filopodia and did not interfere with Map targeting to the mitochondria (Alto, Shao et al. 2006). Substitution of the N-terminal mitochondrial target sequence of map by the N-terminal signal of Tir abolishes mitochondrial localization and tolerates the filopodia formation (Kenny 2002). Map induces filopodia by mimicking GEF activity over the Cdc42 Rho small GTPase, which leads to actin cytoskeletal modification to induce filopodia formation (Huang, Sutton et al. 2009). Map has a TLR domain that allows its interaction with the PDZ1 domain of NHERF1, it is proposed that this induces recruitment of activated Ezrin leading to activation of the small GTPase RhoA and the posterior activation of RhoA/ROCK pathway for stabilization of the filopodia (Simpson, Shaw et al. 2006, Berger, Crepin et al. 2009). EPEC induces a fine modulation of actin cytoskeleton of the cell, as Tir induces filopodia downregulation. Intimin-Tir interaction induces activation of Tir and the recruitment of NcK. The latter has more affinity for N-WASP than Cdc42 and this sequesters N-WASP from the Cdc42-GTP pathway involved in filopodia (Tomasevic, Jia et al. 2007, Berger, Crepin et al. 2009). Additionally, Tir bound to NcK triggers local activation of GTPase-activating proteins (GAPs) and this inactivates Cdc42 (Zhao, Ma et al. 2003). Another mechanism reported for filopodia withdrawal is the release after Intimin-Tir interaction of a GXLR motif in the C-terminal cytoplasmic

region of Tir, which possesses a GAP-like activity and induces the switch-off of Cdc42 with the consequent filopodia downregulation (Kenny 2002). We did short-time infections of Swiss 3T3 cells with EPEC2, EPEC1 and EPEC0 with and without *map* integrated in the chromosome. Through actin staining of infected cells and immunofluorescence microscopy we found that all the strains with *map* in the chromosome were able to induce filopodia formation at the site of microcolony formation (Figure 28). For this assay we synchronized the infection with centrifugation to allow the attachment of EPEC0 at the same time than the rest of the EPEC strains and under these conditions EPEC0+*map* was able to translocate the amount of Map necessary to induce filopodia. Therefore, Map translocated from chromosomal expression was able to induce filopodia without the help of any other effector. Although, the relevance of filopodia formation during *in vivo* infections is not yet known, the study of EPEC effectors that directly and indirectly manipulates Rho GTPases is relevant, because of the vast number of cellular processes that are controlled by the small Rho GTPases (Kenny 2002).

Infecting bacteria alert their presence to the host immune system through bacterial antigens. Hence, to escape from the host immune response, bacterial pathogens have acquired effectors that modulate the host immune response to allow the progression of infection (Takeuchi and Akira 2010). For instance, the OspG from *Shiguella flexneri* is reported to prevent phospho-IκBα degradation and TNF-α-induced activation of NF-κB. It was reported that upon infection of ileal loops in rabbit, an *ospG* mutant induced stronger inflammatory response than the wt strain (Kim, Lenzen et al. 2005). Also, the *Salmonella* SpvC T3 secreted effector is a phosphothreonine lyase that target MAPK in the nucleus. Mice infected with *Salmonella enterica* serovar Typhimurium lacking the *spvC* gene showed pronounced colitis when compared with mice infected with the wild-type strain(Haneda, Ishii et al. 2012).

The A/E pathogens EPEC, EHEC and *Citrobacter rodentium* also are endowed of effector proteins to targets the host immune response (Pearson, Riedmaier et al. 2011, Pham, Gao et al. 2012, Hodgson, Wier et al. 2015). In fact, EPEC

infection is characterize by a week inflammatory response (Dean and Kenny 2009). It has been reported that EPEC translocates in a T3SS-dependet manner NleE, NleB, NleH1 and NleC to disrupt the NF-κB proinflammatory pathway. The NleC EPEC effector protein is widely characterized by disrupting the NF-κB pathway through the degradation of p65 transcription factor. NleC is reported to preferentially degrade active free p65 but it can also degrade IκB bound p65. NleB and NleE through different mechanism disrupt the NF-κB signaling pathway, preventing IκB degradation and the release of p65 from the NF-κB complex, hindering in this way the nucleation of p65. Several studies propose a model in which NleC function downstream of NleB and NleE to degrade the active p65 that scape from the NleB and NleE inhibition (Newton, Pearson et al. 2010, Yen, Ooka et al. 2010, Pearson, Riedmaier et al. 2011). Our results after infecting HeLa cells with EPEC0+*nleC* revealed that the individual T3SS translocation of NleC is enough to decrease the total p65 of the infected cells. And also EPEC1+*nleC* and EPEC2+*nleC* were able to induce degradation of p65. This indicates that *nleC* reintegration in the chromosome allows the appropriate expression of *nleC* and that the three effector mutant EPEC strains translocates the amount of protein necessary to induce p65 degradation. EPECwt induces less degradation of p65 because the wild-type strain is endowed of NleB and NleE to avoid IκB degradation (Figure 29). As NleC degrades free p65 more efficiently, during EPEC-wt infection there is less free p65 to be degraded by NleC. This correlates with previous results (Pearson, Riedmaier et al. 2011) in which EPEC-wt need longer time to induce degradation of p65 than a Δ*nleE* strain. It is significant to highlight that previous studies identified degradation of p65 by NleC with the strategy of loss of the function by the mutant strain and the gain of the function using plasmids complementation and also with ectopic expression of *nleC*. The effector mutant EPEC strains in our study translocate NleC from chromosomal native expression levels , which are sufficient for degradation of p65.

It has been reported that Tir interaction with SPH-2 tyrosine phosphatase enhances their inhibitory association with TRAF6 preventing the NF-κB signaling pathway (Yan, Quan et al. 2013). This results remains controversial because strains lacking *tir* do not adhere well to the cell and this may affect

the translocation of other effectors implicated in the immune response. Our results with EPEC1+*nleC* and EPEC2+*nleC*, both with *tir* in the chromosome and the former translocating high levels of Tir, suggest that Tir is not blocking the NF-κB signaling. Tir blocking the NF-κB pathway should reduce the release of p65 from the NF-κB complex, which should be evidenced by reduction in the p65-NleC degradation. In contrast, we found that the three effector mutant strains with *nleC* reduce the total p65 of the cells at the same level and this suggests that the same level of p65 released from NF-κB complex. All together, these data demonstrate that the effector mutant EPEC strains offer an optimal tool for translocation of physiological amounts of defined effector proteins to investigate EPEC infection.

Infection of human intestinal biopsies using effector mutant EPEC strains

The pathogenic mechanisms of EPEC have been widely investigated through *in vitro* infection of cultured cell lines (Scaletsky, Pedroso et al. 1999, Kanack, Crawford et al. 2005, Nieto-Pelegrin and Martinez-Quiles 2009, Munera, Crepin et al. 2010, Glotfelty, Zahs et al. 2014). However, most cell lines used are nonpolarized and are not from intestinal origin. On the other hand, the study of EPEC infection *in vivo* is hindered because EPEC is a human-specific pathogen (Schuller, Chong et al. 2007). A surrogate model established to investigate the A/E pathogens infection *in vivo* is the mice infection by the mouse pathogen *Citrobacter rodentium.* A study with *Citrobacter* (Deng, Vallance et al. 2003) demonstrated that *Citrobacter* infection of cells *in vitro* requires Tir phosphorylation for actin-pedestal formation but Tir-phosphorylation deficient mutants still colonize the mouse gut and induce A/E lesion and crypt hyperplasia. This highlights the necessity to use a model for EPEC infection closer to the *in vivo* conditions. A good established model to study EPEC infection is the infection of *in vitro* cultured human intestinal biopsies (IVOC), which allows the formation of A/E lesions undistinguishable from those observed *in vivo* in biopsies of patients with diarrhea by EPEC (Knutton, Lloyd et al. 1987, Hill, Phillips et al. 1991). Interestingly, it has been reported that EPEC strains deficient of phosphorylation of Tir Y474 and Y454 are able to induce the A/E lesion formation in the IVOC infection in

an NcK-independent manner while the phosphorylation at these positions is crucial to induce the pedestal formation during *in vitro* infections of cultured cells lines (Schuller, Chong et al. 2007). In our work the infection of human duodenal biopsies by the effector mutant EPEC strains revealed that, although EPEC2 and EPEC1 strains were able to induce actin-pedestals *in vitro*, none of the infected biopsies with these strains showed A/E lesion, see Table 8 and Figure 30. Thus, Tir is not sufficient to induce A/E lesion and other effector besides Tir and EspZ are needed to induce the A/E lesion in intestinal surfaces. We further used the IVOC assay to characterize whether LEE or non-LEE effector(s) were involved to induce the A/E lesion. Infection of human biopsies with EPEC2-LEE+ (carrying all LEE effectors) did not reveal A/E lesions whereas infection with EPEC9 (*espZ*, *tir* and all the non-LEE effectors) induced A/E lesions in biopsies with efficiency as high as the wild-type EPEC strain, see Table 8 and Figure 30. Therefore, non-LEE effectors are essential to induce A/E lesion in human intestinal tissue. It was previously reported that LEE confers the attaching and effacing phenotype to *E. coli* K-12 strains and it was observed by the A/E lesion formation in Caco-2 cells *in vitro* (McDaniel and Kaper 1997). Contrary, our results indicate that additional effectors are required for the A/E lesion formation and further reveal the importance of the IVOC assay to analyze effectors roles during infection.

Most of the non-LEE effectors are implicated in counteracting the host immune response like NleB, NleE, NleC, NleD, NleH1, LifA and NleF. Although *in vivo* infection is the ideal situation to study the complete immune response against an infection, some studies have used intestinal biopsies tissues to analyze some immune response traits. It has been reported with an organ culture system using human intestinal biopsies that after stimulation, there is epithelial infiltration and lamina propia T-cell activation (Auricchio, Paparo et al. 2004). Another studies evaluated the innate immune response of cultured human duodenal biopsies using a polarized IVOC system for optimal of bacterial-host cell interaction. Using this system they showed that apical EPEC infection of duodenal mucosa results in increased IL-8 mRNA and protein expression and that it was mainly dependent of flagellin and Toll like receptor 5 interaction (Schuller, Lucas et al. 2009). Therefore, we

speculated that non-LEE effectors could be necessary to block the immune response of the intestinal biopsies to enable the A/E lesion formation during *ex vivo* EPEC infection.

With the aim of characterizing non-LEE effectors involved in A/E lesion, we infected human biopsies with EPEC8, EPEC7 and EPEC6; these strains have sequential deletion of effectors genes present in IE5, IE6 and IE2, respectively (Table 9). While IVOC infection with EPEC8 show efficient A/E lesion formation, infections with EPEC7 and EPEC6 reduced the efficiency of A/E lesion to 54% and 23% of the infected samples, respectively. The low efficiency induced with EPEC6 tempted us to analyze the contribution of IE6 and IE2 encoded effectors. These two IEs encode a similar set of effectors, IE6 (*espL, nleB1, nleE1* and *efa1/lifA*) and IE2 (*espL*, nleB*, nleE2* and *efa/lifA*-like) (Figure 31A). The *espL** and *nleB** effectors of IE2 are pseudogenes, which suggests that are *nleE* and/or *efa1*/*lifA* homologs the effectors playing a major role in A/E lesion formation. NleE2 has an internal deletion of 56 residues that could impede T3-translocation into HeLa cells (Nadler, Baruch et al. 2010). Efa1/LifA-like has been reported to be T3-secreted by a hypersecreting EPEC mutant strain although there is no evidence of its translocation into HeLa cells (Iguchi, Thomson et al. 2009, Deng, Yu et al. 2012). To investigate whether NleE2 or Efa1/LifA-like were responsible of the reduction in the A/E lesion formation by EPEC6, we generated the mutant strains EPEC7Δ*nleE2* and EPEC7Δ*lifA*-like (Figure 31A). Since EPEC7Δ*lifA*-like, and not by EPEC7Δ*nleE2*, reduced the efficiency of A/E lesion formation of EPEC7, we propose that *lifA*-like is a non-LEE effector that has a major contribution for efficient A/E lesion formation by EPEC (Table 9).

The *lifA*-like was first described in the genome of EPEC O127:H6 strain E2348/69 as a homolog of *lifA* with 28% aa identity with Lymphostatin (LifA) (Iguchi, Thomson et al. 2009). The *lifA* was first described in EPEC O127:H6 as a chromosomally encoded protein with a predicted molecular mass of 365 kDa, it has been shown to be translocated into mammalian cells in a T3SS-dependent manner (Klapproth, Scaletsky et al. 2000, Deng, Yu et al. 2012). LifA homologs are found in the genome of A/E pathogens,

LifA in *Citrobacter rodentium*, Efa1 in non-O157 EHEC strains, and the less homologous ToxB encoded by a pO157 plasmid in EHEC O157:H7 strain (Klapproth, Scaletsky et al. 2000, Abu-Median, van Diemen et al. 2006). LifA and Efa1 are nearly the same protein with 99% of aa identity, therefore they are named *efa1/lifA* (Deng, Yu et al. 2012).

The N-terminus of Lymphostatin bears significant homology to the N-terminal catalytic domain of large clostridial toxins (LCTs) that contains a glycosyltransferase domain (Busch, Hofmann et al. 1998, Klapproth, Scaletsky et al. 2000). LifA, Efa1 and ToxB hold three distinct motifs: a glycosyltransferase motif, a cysteine protease motif and an aminotransferase II motif (Klapproth, Sasaki et al. 2005, Babbin, Sasaki et al. 2009, Deacon, Dziva et al. 2010). Efa1/LifA homologs have been implicated in blocking lymphocyte proliferation and activation of immune response, as adhesins and colonization factors, and inducing intestinal barrier disruption by manipulation of cellular Rho GTPases (Klapproth, Scaletsky et al. 2000, Badea, Doughty et al. 2003, Klapproth, Sasaki et al. 2005, Babbin, Sasaki et al. 2009, Deacon, Dziva et al. 2010). Given the different proposed roles of LifA homologs in A/E pathogens, they have been considered multitalented virulence factors (Klapproth and Meyer 2009). Interestingly, Efa1/LifA-like protein has the three conserved motifs found in Efa1/LifA, despite being a smaller protein with 30% of aa identity (Deng, Yu et al. 2012). Although, the coding region for the first 50 aa of this protein fused to Bla was not translocated into HeLa cells, we speculate that it could be an alternative mechanism of translocation for this protein independent of its N-terminal region. Some proteins could be secreted in a T3-depedent manner to the environment of the infection and play a role in the pathogenesis once in contact with the epithelial cells (Deng, Yu et al. 2012). While *in vivo* infection with EHEC and *Citrobacter rodentium* in calves and mice respectively, confirmed the important role of Efa1/ LifA in intestinal colonization (Klapproth, Sasaki et al. 2005, Deacon, Dziva et al. 2010), *in vivo* studies to evaluate the role of LifA/Efa1 during EPEC infection have not been reported so far. For the best of our knowledge, this is the first study that implicates EPEC *efa1/lifA* homologs as the major non-LEE virulence factors assisting A/E lesion formation in human intestinal tissues *ex vivo*. Despite the major

role unveiled for Efa1/LifA homologs in A/E lesion formation, our study also shows that these proteins are not essential for this process, since EPEC6 stills induces A/E lesion formation in ca. 23% of infected biopsies. This indicates that non-LEE effectors encoded in PPs also participate in assisting Tir and EspZ for A/E lesion formation. Together these results demonstrate an essential role of non-LEE effectors in the induction of the A/E lesion in *ex vivo* infections of human intestinal tissues by EPEC. Various non-LEE effectors contribute for efficient A/E lesion formation in an additive manner, being Efa1/LifA homologs major contributors, but not essential for this process.

Potential applications of the effector mutant EPEC strains

The effector mutant EPEC strains are a useful biological tool to study the role of effectors in EPEC pathogenesis. Using these mutant strains we can translocate an individual effector or defined combination of effectors using *in vitro* infections of conventional nonpolarized culture cell lines, polarized cell lines of intestinal origin (e.g., Caco-2 and T84 cells), and human intestinal biopsies. EPEC is human enteric pathogen with a narrow host range, but some *in vivo* animal models are available for research. The larvae *of Galleria mellonella*, which possess like mammals a complex innate immune system, could be an interesting and simple model to evaluate the virulence of our effector mutant EPEC strains. It has been reported that bacterial strains attenuated in mammalian models demonstrate lower virulence in *Galleria* (Ramarao, Nielsen-Leroux et al. 2012). Also, it has been reported that EPEC strains defectives in T3SS lose their lethal effect in *Galleria* (Leuko and Raivio 2012). Infection of mammalian models is important to evaluate the effect of innate and adaptive immune host-answer as well as components of the microbiota, which all together have protective role against A/E pathogens infection (Law, Gur-Arie et al. 2013). The oral infection with EPEC of neonate mice has been reported to induce an effective intestinal colonization associated with the generation of A/E lesion-like focal microcolonies in the intestinal epithelial surface, as well as mucosal innate immune stimulation, which were dependent on the presence of EPEC virulence factors (i.e., BFP and T3SS) (Dupont, Sommer et al. 2016). Therefore, oral infection of

newborn mice will be a relevant approach to evaluate *in vivo* the effector mutant strains and strains with defined effectors.

Most effector mutant EPEC strains are likely to be strongly attenuated, whereas they maintain intact the external antigenicity of the wild-type strain. A mutant strain with a functional T3SS and the minimum set of effectors necessary to colonize and attach to the intestine surface, can be used to induce protection against EPEC but not the acute diarrhea that characterize EPEC infection. Safe EPEC mutant strains with ability to colonize and attach to the human intestine can compete with the commensal microbiota for intestinal nutrient and niches and we can engineer them to translocate heterologous bacterial protein to generate immunity against other enteric pathogen such as: *Vibrio cholera* and *Shigella spp*. Studies illustrate that bacterial outer membrane proteins are ideal molecules as vaccine antigens. The outer membrane protein A (OmpA) of *Shigella flexneri* 2a induces humoral and cellular immune response against *Shigella spp*. in mice (Pore and Chakrabarti 2013, Pore and Chakrabarti 2016). The B subunit of cholera toxin (CTB) is a potent immunogen associated with protection against *V. cholera* (Price, McFann et al. 2013). The advantage of using CTB is that stimulation of the antitoxic immunity is complete safety, without the risk of the reversion of the toxoid (chemically-inactivated-toxin) (Levine, Kaper et al. 1983). Thus, a vaccine based in killed whole cells of *V. cholera* plus recombinant cholera toxin B subunit has been used for human immunization against *V. cholera* (Organization 2010). Regarding EHEC enteric pathogens, immunogenic protection induced by selected protein(s) of EHEC has been reported, such as: Efa1, Intimin, EspB and the B subunit of the Shiga toxin 2 (Stx2)(Marcato, Griener et al. 2005, Szu and Ahmed 2014, Riquelme-Neira, Rivera et al. 2015, Rabinovitz, Larzabal et al. 2016). Thus, genome engineering to drive expression these immunogenic proteins by the effector mutant EPEC strains will be a good approach for vaccine development to these and other enteric pathogen.

The effector mutant strains can also be potential therapeutic tools for the treatment of intestinal inflammatory and autoimmune disorders. Patients with inflammatory bowel diseases (IBDs), including Crohn´s disease (CD)

and ulcerative colitis (UC), are characterized by abnormal activation of the immune system in the gut, resulting in chronic inflammation of the digestive system (Martin, Miquel et al. 2013). Recombinant lactic acid bacteria (LAB) have been engineered to express immunomodulatory molecules for the treatment of IBDs. Secretion of the anti-inflammatory cytokine IL-10 by the recombinant *Lactococcus lactis* induced beneficial effect in mouse models of IBD (Steidler, Hans et al. 2000). A phase II clinical trial with *L. lactis* expressing IL-10 revealed that although safety, tolerability and environmental containment have been achieved, no statistically significant difference has been found versus placebo in beneficial effects (Martin, Miquel et al. 2013). Due to this outcome, a good approach will be optimization of the delivery system. Therefore, a good possibility will be to use recombinant effector mutant EPEC strains having good colonization to translocate the IL-10 cytokine into the enterocytes of patients with IBDs. This can be extended to the translocation of defined effectors downregulating the immune response (e.g. acting on NF-kB signalling). In summary, we anticipate interesting applications of the effector mutant EPEC strains generated in this PhD thesis in basic research, vaccine development, and therapeutic interventions against autoimmune disorders in the gastrointestinal tract.

CONCLUSIONS

1. We have deleted the whole repertoire of T3SS effectors found in the genome of EPEC serotype O127:H6 strain E2348/69 through a markerless gene deletion strategy. Deletions were done from the start codon to the stop codon of individual ORFs or cluster of effectors, maintaining the assembly of a functional T3SS injectisome and without affecting growth, viability and cellular morphology of the bacteria.

2. All the effector mutant strains carrying Tir are able to trigger actin polymerization in pedestal-like structures underneath bacteria attached to HeLa cells. All effector mutant strains and the effector-less strain (EPEC0) translocate substrates of the T3SS (i.e., $EspF_{1\text{-}20}$-Bla) into HeLa cells.

3. Deletion of the myosin interacting domain (mid) of EspB translocator protein does not disrupt T3SS-protein translocation but dramatically reduces translocation levels.

4. The strain harboring Tir and lacking EspZ (EPEC1) translocates higher levels of T3SS substrates and induces higher cytotoxicity in HeLa cells. The absence of EspZ in EPEC1 leads to a higher level of Tir translocation into HeLa cells than that of EPECwt. The translocation of Tir by EPEC2, expressing Tir and EspZ, is lower than that of EPECwt, suggesting that additional effector(s) may be necessary to stabilize Tir in HeLa cells.

5. Following a markerless gene integration strategy, a single copy of gene-encoding effectors *espH*, *map*, and *nleC* were integrated in their native loci within LEE and PP4 of effector mutant strains EPEC2, EPEC1 and EPEC0. The individual translocation of EspH, Map and NleC from single chromosomal gene copy and native gene expression induced the reported phenotypes of focal adhesion disassembly (EspH), filopodia formation (Map) and p65 degradation (NleC). Therefore, the effector mutant strains are useful to study the phenotype caused by individual effectors in the context of EPEC infection and physiological expression levels of the effectors.

6. Strains expressing Tir (EPEC1) or Tir and EspZ (EPEC2) effectors alone were not able to induce A/E lesions in human intestinal biopsies, despite

inducing actin pedestal-like structures in HeLa cells. Hence, Tir effector is required but not sufficient to induce A/E lesions in *ex vivo* infections of human intestinal tissues.

7. EPEC requires the expression of a subset of non-LEE effectors to induce A/E lesion in human intestinal biopsies. The EPEC effector mutant strain maintaining all six LEE effectors and devoid of all non-LEE effectors (EPEC2-LEE^{+}) was unable to induce A/E lesions in human intestinal biopsies.

8. Non-LEE effectors encoded in IE6, IE2 and PPs have an additive role in augmenting the efficiency of A/E lesion formation in human intestinal biopsies. Deletion of IE6 and IE2 encoded effectors (EPEC6) dramatically reduces the efficiency of the A/E lesion formation from wild-type levels (ca. 76%) to ca. 54% in EPEC7 (ΔIE6) and ca. 23% in EPEC6 (ΔIE6ΔIE2). The Efa1/LifA homologs encoded in IE6 and IE2 are the major contributors for the development of efficient A/E lesions in human intestinal tissues. In the absence of Efa/LifA homologs, the effectors encoded in PPs, together with Tir and EspZ, still allow A/E lesion formation by EPEC at low efficiencies (ca. 23-33%).

1. Hemos delecionado todo el repertorio de efectores del T3SS en el genoma del EPEC serotipo O127:H6 cepa E2348/69 a través de una estrategia de deleción libre de marcas. Las deleciones fueron hechas desde el codón de inicio hasta el codón de finalización de las ORFs individuales o grupos de efectores, manteniendo el correcto ensamblado de los inyectisomas del T3SS y sin afectar al crecimiento, la viabilidad o la morfología de las bacterias.

2. Todas las cepas mutantes en efectores que llevaban Tir son capaces de desencadenar la polimerización de actina en estructuras en forma de pedestal bajo las bacterias unidas a células HeLa. Todas las cepas mutantes en efectores y la cepa sin efectores (EPEC0) translocan sustratos del T3SS (ej. $EspF_{1\text{-}20}$-Bla) al citoplasma de células HeLa.

3. La deleción del dominio de interacción con miosina (mid) de la proteína translocadora EspB no impide la translocación de proteínas por el T3SS pero reduce drásticamente los niveles de translocación.

4. La cepa que presenta Tir y carece de EspZ (EPEC1) transloca mayores niveles de substratos del T3SS e induce mayor citotoxicidad en células HeLa. La ausencia de EspZ en EPEC1 conlleva niveles de translocación de Tir a células HeLa mayores que los producidos por la cepa EPECwt. La translocación de Tir en la cepa EPEC2, que expresa Tir y EspZ, es menor que la del EPECwt, lo que sugiere que otros efectores podrían necesitarse para estabilizar Tir en células HeLa.

5. Siguiendo una estrategia de integración libre de marcas, una copia única de los genes codificantes de los efectores *espH*, *map* and *nleC* fueron integrados en su sitio original en el LEE y en el PP4 de las cepas mutantes en efectores EPEC2, EPEC1 y EPEC0. La translocación individual de EspH, Map y NleC desde una copia única en el cromosoma y bajo regulación nativa indujo los fenotipos previamente reportados de desensamblado de las adhesiones focales (EspH), formación de filopodios (Map) y degradación de p65 (NleC). Por lo tanto, las cepas mutantes en efectores son útiles para estudiar el fenotipo causado por efectores individuales en el contexto de la infección de EPEC con niveles de expresión fisiológicos de los efectores.

6. Las cepas expresando únicamente los efectores Tir (EPEC1) y Tir y EspZ (EPEC2) no fueron capaces de inducir lesiones A/E en biopsias intestinales humanas, a pesar de inducir la formación de estructuras en forma de pedestales de actina en células HeLa. Así, Tir es necesario pero no suficiente para inducir la formación de la lesión A/E durante la infección *ex vivo* de tejidos humanos intestinales.

7. EPEC necesita la expresión de un subconjunto de efectores no-LEE para inducir la lesión A/E en biopsias intestinales humanas. La cepa mutante en efectores que mantiene los seis efectores del LEE y está desprovista de todos los efectores no-LEE (EPEC2-LEE^{+}) fue incapaz de inducir la formación de la lesión A/E en biopsias intestinales humanas.

8. Los efectores no-LEE codificados en IE6, IE2 y PPs presentan un papel aditivo para aumentar la eficiencia en la formación de la lesión A/E en las biopsias intestinales humanas. La deleción de los efectores codificados en IE6 y IE2 (EPEC6) redujo dramáticamente la eficiencia de la formación de la lesión A/E, desde niveles de la cepa EPECwt (aprox. 76%) a aprox. 54% en el EPEC7 (ΔIE6) y aprox. 23% en el EPEC6 (ΔIE6ΔIE2). Las proteínas homologas a Efa1/LifA codificadas en IE6 y IE2 son las que contribuyen principalmente al desarrollo eficiente de la lesión A/E en tejidos humanos intestinales. En ausencia de las proteínas homologas Efa1/LifA, los efectores codificados en los PPs, junto a Tir y EspZ, aún permiten la formación de la lesión A/E por EPEC pero con una baja eficiencia (aprox. 23-33%).

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