# Regulation of the LEE-pathogenicity island in attaching and effacing bacteria

**C. Lara-Ochoa, R. Oropeza and A. Huerta-Saquero**

Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad 2001. Cuernavaca, Morelos, México.

Diarrhoeagenic *E. coli* strains are a major health problem worldwide: in developing countries they are one of the principal causes of morbidity and mortality among young children, while in industrialized countries are often responsible of food-poisoning outbreaks. EPEC (Enteropathogenic *E. coli*) and EHEC (Enterohaemorrhagic *E. coli*) are two important *E. coli* pathotypes that share the ability to cause the attaching and effacing (A/E) lesion, characterized by the intimate adherence of bacteria to actin-rich pedestal like structures on the surface of intestinal epithelial cells. The genetic determinants involved in the development of this phenotype are mainly located in the Locus of Enterocyte Effacement (LEE), a 35-43 Kb pathogenicity island coding for a type III secretion system (T3SS), translocator and effector proteins, chaperons and transcriptional regulators. This review briefly describes the molecular mechanisms involved in the development of the A/E lesion and summarizes the current knowledge on LEE transcriptional regulation, mainly focusing on the antagonic role played by the histone-like protein H-NS and its paralogue the LEE-encoded regulator Ler on silencing and antisilencing; the role of GrlR and GrlA mutual interaction in negative and positive regulation; the involvement of strain specific accessory regulators such as PerC, EivF, EtrA, GrvA, and RegA; as well as of other global regulators .

**Keywords**: Pathogenic *E. coli*; virulence gene regulation; A/E pathogens; Locus of Enterocyte Effacement.

## 1. A/E pathogens

*Escherichia coli* is a facultative anaerobic bacterium, commonly found as a resident member of the intestinal microbiota living at the colonic mucus layer, where they play an important role in the intestine physiology [1]. Moreover, *E. coli* has metabolic and regulatory capabilities enabling it to get adapted as a free-living bacterium [reviewed by [2]].

Since *E. coli* and *Salmonella* diverged from a common ancestor one hundred million years ago, *E. coli* strains have evolved and diversified by acquiring new genetic information through numerous horizontal transfer events. These events provided virulence attributes that allowed *E. coli* variants to adapt and exploit different host niches using diverse molecular mechanisms [3]. Most *E. coli* infections occur on mucosal surfaces, although some strains can colonize or invade other niches in the human body.

Diarrhoeic diseases are a public health problem around the world, particularly in emergent countries. Around 2.5 millions children under 5 years died each year as a consequence of diarrhoeal diseases [5]. Based on their virulence mechanisms and the clinical syndromes they caused, diarrhoeagenic *E. coli* strains are grouped into six major categories called pathotypes: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffuse adherent *E. coli* (DAEC) [4, 6].

The colonization mechanisms performed by the *E. coli* pathogenic strains are designed to cope with host natural barriers such as: low pH, mucus, antimicrobial enzymes and peptides, peristalsis and competing microflora, which includes non-pathogenic *E. coli* strains [7]. Upon colonization, diarrhoeagenic *E. coli* strains display a myriad of virulence mechanisms that ultimately cause disease.

Diarrhoea is the result of an alteration in ions and solutes absorption and secretion throughout the intestinal epithelium, followed by water movement in order to recover optimal ion concentrations [9]. In spite of the advances in knowledge of A/E bacteria pathogenesis at cellular and genetic level, the diarrhoea physiopathology is still poorly understood. Dramatic loss of microvilli at intestinal epithelium and consequent diminution of absorption surface, certainly contribute to diarrhoea generation. Diarrhoea induced by A/E pathogens constitutes a mechanism that confers a competitive advantage over normal intestinal flora, as once intimate attachment is established on the surface of infected enterocytes, bacteria remain attached to the intestinal surface whereas other bacteria with poor colonization abilities may be flushed out by the intestinal flow [reviewed by [10]].

Intimate adherence is the final step of an interesting virulence mechanism developed by several pathogenic microrganisms named as "A/E" pathogens; this name refers their ability to induce a unique and typical histopathological lesion known as the attaching and effacing lesion [reviewed by [6] ]. A/E pathogens are also named attaching and effacing *E. coli* (AEEC), including *E. coli* strains previously classified as EPEC and EHEC; however in 2000 was reported that *Citrobacter rodentium*, a mice pathogen, also causes A/E lesion [11]. Besides human and mice, A/E pathogens infect rabbits, pigs, dogs, cats and chickens [reviewed by [4] ].

**Enteropathogenic *E. coli* (EPEC).** It was the first group of *E. coli* strains reported as the causal agent of diarrhoea outbreaks in children from developing countries by 1940 to 1950. It is the representative strain in the A/E pathogens family [reviewed by [4] ]. It was named EPEC by Neter in 1955, emphasizing neither produces toxins or invades [reviewed by [12] ]. Nowadays, it is one of the main etiologic agents causing diarrhoea that affects child younger than 6

months old all around the world, but most importantly in emergent countries [reviewed by [4] ]. The EPEC type strain studied in many laboratories is designated E2348/69 (O127:H6), it was isolated from an outbreak in Taunton, England on 1969 [reviewed by [4] ]. The main symptom of the disease caused by EPEC is acute watery diarrhoea of diverse severity, also there are reports of persistent diarrhoea [13]. Besides diarrhoeic episodes, other symptoms are vomit, fever, and fluids loss [4, 14]. EPEC colonizes the small intestine and can cause diarrhoea in healthy adults when it is given in high doses ($10^8$ to $10^{10}$). However, it is considered that the natural infective dose is lower than that one [reviewed by [4] ].

**Enterohaemorrhagic *E. coli* (EHEC)**. This strain was originally described by two clinical observations: 1) it is an *E. coli* with an uncommon serotype, O157:H7, causing haemorrhagic colitis, a severe gastrointestinal disease; 2) there was a correlation between the haemolytic uremic syndrome (HUS) and toxins production by *E. coli*. Those fundamental considerations allowed the discovery of a new kind of enteric pathogen, enterohaemorrhagic *E. coli*, recognized on 1982 as causative of human disease [reviewed by [15]]. There are several *E. coli* serotypes producing "Shiga" toxin (Stx), able to cause bloody diarrhoea, haemorrhagic colitis and haemolytic uremic syndrome in humans [4, 16, 17]. EHEC strains, particularly those belonging to serotype O157:H7, have been isolated in epidemic outbreaks of intestinal diseases all around the world, mainly in industrialized countries [18]. The major EHEC natural reservoir is the bovine and ovine intestine that is why the first EHEC outbreaks were due to uncooked meat consumption. EHEC infection occurs by ingesting contaminated water or food, with a few person-to-person infections reported until now. The infectious dose of EHEC is thought to be extremely low [17]. EHEC´s most important virulence characteristic is the production of Verotoxin or Shiga toxins (STX1 or STX2). Toxin production defines whether the disease is localized or systemic. The localized one is characterized by bloody diarrhoea caused by the toxin getting internalized in the large intestine cells. When toxin enters into bloodstream, binds endothelial cell receptors at brain and kidneys, produces the HUS, a serious disease in child younger than 5 years old [17, 19]. HUS is characterized by haemolytic anaemia, thrombocytopenia and acute renal failure. Kidneys are the most damaged organs as a consequence of HUS, this disease is fatal in 3-75% of the events and 30% of survivors show chronic renal failure, hypertension and neurological damage [reviewed by [19]].

***Citrobacter rodentium* (CR).** This bacterium is a mouse natural pathogen, causing transmissible colonic hyperplasia, characterized by the reversible hyperproliferation of colonic cells, diarrhoea and rectal prolapse [11] . *C. rodentium* is genetically related to *E. coli*, becoming a good model to study the *in vivo* molecular mechanisms involved in pathogenesis and A/E lesion formation, both from the pathogen and the host perspective [20].

### 1.1 Attaching and effacing (A/E) lesion.

A/E lesion is characterized by microvillus destruction at the enterocyte brush border and cytoskeleton rearrangements; actin is accumulated and polymerized leading to the formation of typical structures seemed to a cup or pedestal, through which the bacteria are intimately attached to the intestine epithelial cells [12, 21, 22]. Pedestal-like structures are 10-μm height above the epithelial cell, also resembling pseudopods [23]. At the beginning, all the histopathologic descriptions on A/E lesion were performed by scanning electron microscopy. On 1989, Knutton and cols. developed a new method named florescence actin staining (FAS). This method confirmed actin site-specific accumulation beneath adhered bacteria, becoming a marker for the A/E lesion [24, 25]. Since then, FAS is a specific diagnostic method to detect bacteria causing the A/E lesion.

Some other cytoskeletal proteins, besides F-actin, are part of the A/E lesion such as: α-□actinin, talin, ezrin, VASP, WASP, myosin light chain and the Arp 2/3 complex [reviewed by [4, 26]]. At histopathologic level, intestinal lesions produced by different A/E pathogens are similar, the only difference is the place in the intestine where they are found: EPEC colonizes the small intestine but EHEC and *C. rodentium* colonize the large intestine; some A/E lesions have been reported at the stomach [reviewed by [27] ].

A/E lesions are instrumental promoting successful colonization, dissemination and bacterial multiplication giving as a result the infection. It has been characterized that pedestals induced by EPEC are dynamic and flexible structures. Moreover, when EPEC is adhered to the pedestal, they move as fast as 0.03-0.07 μm/s on host cell surface [reviewed by [28]], suggesting this event could promote bacterial dissemination [reviewed by [29] ].

A/E pathogens are an example of host-pathogen interaction, where the pathogen has evolved mechanisms to hijack the host cell machinery to ensure survival. Contrary to *Salmonella, Shigella* and *Listeria*, A/E pathogens are extracellular pathogens [30].

## 2. Genetic organization of LEE Pathogenicity Island

A/E lesion formation requires the coordinated expression of LEE PAI genes. EPEC LEE PAI is necessary and sufficient for A/E lesion formation [Reviewed by [26, 31]].

In spite of differences in size and genetic content between A/E pathogens, LEE PAI maintains a basic structure. It includes 41 genes organized mainly in 5 polycistronic operons: *LEE*1, *LEE*2, *LEE*3, *LEE*4, *LEE*5; two bicistronic operons: *espG-rorf1* and *grlRA*; and 4 monocistronic entities: *rorf3, cesF, map* and *escD* (Fig 1). *LEE1*, *LEE2* and

 

*LEE3* operons encode for a type three-secretion system (TTSS), necessary to translocate bacterial proteins towards the enterocyte [32-34]. *LEE*4 contains *esp* genes (EPEC secreted proteins), *espADB* encodes for translocator proteins that form a conduit through which the TTSS delivers effector proteins to the host cell [35]. Genes necessary for bacterium-host cell intimate adhesion are located in *LEE*5: *eae* gene encodes for the adhesin intimin, *tir* for the Translocated Intimin receptor (Tir), and *cesT* for the Tir chaperone [32, 33, 36].

Ler, the first gene encoded in *LEE*1, is the master regulator of LEE PAI. Besides Ler, other regulator proteins are encoded in LEE PAI: GrlR (that acts like a negative regulator) and GrlA (a positive regulator). Genes encoding for those proteins are organized as the *grlRA* operon (Fig 1) [37, 38].

Other genes encoding for chaperones and effector proteins involved in subversion of several host pathways are distributed throughout the LEE PAI, as well as in prophages and other PAIs located throughout A/E pathogens chromosome.

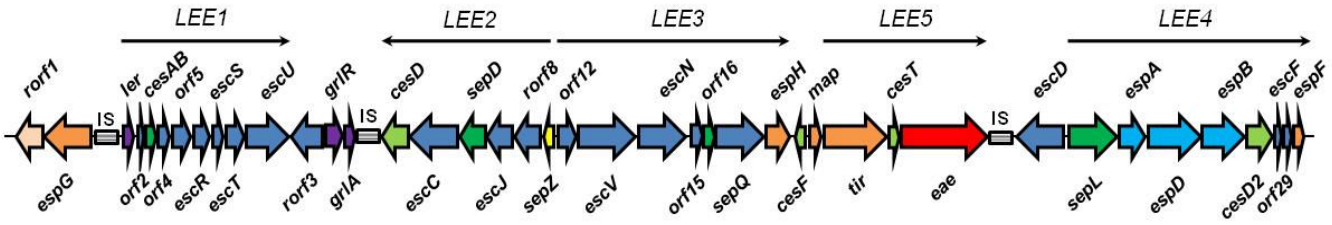


**Fig. 1**. Genetic organization of EPEC LEE pathogenicity island [39].

2.1 Model of A/E lesion development

The development of A/E lesion consists of 3 stages: 1) Localized adherence, 2) Signal transduction and 3) Intimate attachment. Donnenberg and Kaper proposed this model for EPEC in 1992.

**1) Localized adherence.** This stage is essential for the signalling processes leading to A/E lesion formation. EPEC harbours plasmid EAF (EPEC adherence factor) [reviewed by [4, 6]]. In this plasmid is located *bfp* operon encoding for type IV pilus BFP (Bundle-forming pilus), which is responsible for bacterial aggregation and microcolonies formation that confer to EPEC adherence capacity to the intestinal epithelial cells [reviewed by [7]]. Nevertheless, EHEC and *C. rodentium* do not posses EAF plasmid neither expresses the BFP. It has been reported that some proteins encoded in LEE PAI are able to promote bacterial adhesion to the host cell [reviewed by [27]]. One of these proteins is EspA that forms a filamentous structure functioning as an extension of the TTSS of A/E pathogens. EspA filaments are required in early stages of A/E lesion formation, reason why an adhesin role is attributed to it. Furthermore, in the absence of BFP (in atypical EPEC strains), EspA promotes, although in lesser degree than BFP, the initial bacterium-host cell interaction [reviewed by [26, 40]].

**2) Signal transduction.** Once bacteria adhere to epithelial cell surface, alteration of eukaryotic signal transduction pathways is induced. Proteins responsible for this phenomenon are denominated effectors and they are codified mainly in the LEE PAI. Effector proteins such as EspG, EspH, EspF, EspZ, EspB, Map and Tir are injected to epithelial cells through T3SS. Nevertheless, proteins encoded outside LEE PAI are also secreted through this apparatus: Cif, NleA/EspI, EspJ and EspFu [reviewed by [41]]. Effects induced in enterocyte by effector proteins entail to A/E lesion formation, with consequent microvillus destruction in the adhesion zone and actin reorganization leading to pedestals formation. Such structures confer to A/E pathogens the advantage to remain as extracellular bacteria avoiding to be dragged by the imminent consequence of their pathogenicity, the diarrhoea [reviewed by [26]]. As a consequence of A/E pathogens infections, there are other effects in eukaryotic cell such as: 1) intestinal barrier function alteration and permeability increase at tight junctions, 2) mitochondrial membrane potential loss, 3) cell cycle inhibition at phase transition G2/M and, 4) cellular apoptosis induction [reviewed by [41]].

**3) Intimate attachment.** Intimate attachment depends of intimin, a bacterial outer membrane protein and Tir, its receptor protein that is translocated to the eukaryotic cell and is located in the plasmatic membrane. Besides interacting with Tir, it has been proposed that intimin interacts with eukaryotic cell receptors such as β1-integrins and nucleolin [reviewed by [26]; [30]]. This is an extraordinary example of a pathogen that provides its own receptor to assure a successful interaction with the host cell [reviewed by [10]]. Once inserted in the plasmatic membrane of enterocyte, Tir adopts a hairpin structure, with an extracellular domain interacting with intimin. Whereas Tir amino- and carboxi-terminals reside in the host cell cytoplasm interacting with cytoskeleton proteins and diverse components of signalling pathways [reviewed by [42]]. Tir amino-terminal domain interacts with diverse focal adhesion proteins such as: α-actinin, talin and vinculin. The function of these proteins is to maintain the actin cytoskeleton-membrane connection. It

has been reported that Tir amino-terminal domain is not required for pedestal formation. However, the carboxi-terminal region is essential for A/E lesions formation [reviewed by [42]]. Hence, Tir has mainly 3 functions: 1) it works as the intimin receptor to establish intimate attachment, 2) it acts like an essential mediator in subversion of signalling cascades within the host cell, and 3) it works as a transmitter of additional signals for the infection development once interaction with intimin has been established [reviewed by [43]].

## 3. Regulation of LEE virulence genes

Genetic determinants of bacterial pathogenicity are subjected to strict regulation that assures expression only under optimal environmental conditions, avoiding at the same time, intense metabolic cost and/or to alert the host immunological system, in order to ensure a successful colonization [reviewed by [12]]. Coordinated expression of virulence genes is controlled by regulatory proteins, which activate or repress particular genes precisely depending on the infection stages. In that sense, the LEE PAI, like other PAIs, also contains genes encoding for regulatory proteins that modulate expression of virulence genes encoded in this island and, interestingly, other genes located outside the LEE PAI [44]. LEE expression is not subjected solely to the action of LEE-encoded regulators, but it is also influenced by global regulators encoded outside LEE PAI [44]. A/E lesions formation is an interesting example of successful spatio-temporal LEE expression in A/E pathogens. Nevertheless, this is not a simple phenomenon, LEE regulation is a very complex process governed by at least 4 types of regulatory influences: 1) environmental factors, 2) a local hierarchy of regulators encoded on LEE PAI, including the positive regulator Ler and the GrlR-GrlA regulatory system, 3) a system of global regulators of *E. coli* that includes H-NS, IHF, FIS, Quorum sensing, SOS response, and two-component systems; and 4) regulators acquired by horizontal gene transfer: PerC, EivF, EtrA, GrvA, RegA [reviewed by [45]]; [46].

### 3.1 Environmental factors and LEE gene expression

Expression and secretion of virulence determinants encoded in the LEE PAI are affected by diverse environmental factors that include the bacterial growth phase, temperature, cations presence, pH, osmolarity, stress conditions and nutrients starvation [reviewed by [12, 47]]. The optimal conditions for the A/E lesions formation are those existing in the intestine or those mimicking them [48]. Therefore, A/E pathogens can be cultured in the laboratory under inducing conditions: DMEM (Dulbecco´s modified Eagle´s Medium), at exponential growth phase, 37°C, pH 7 and, physiological osmolarity; these are optimal conditions for the expression and secretion of virulence proteins encoded in LEE PAI [48, 49]. Also, it has been determined that virulence protein secretion is dependent on the presence of $NaHCO_3$ (sodium bicarbonate) [48, 50]. Moreover, bicarbonate is sufficient to promote virulence determinants secretion in rich culture conditions, like LB (Luria-Bertani broth) [51], where LEE genes expression is generally undetectable (repressing growth condition). $HCO_3$- ion is present at high concentrations in pancreatic juice and it is released by intestinal mucosa in the large intestine, in order to neutralize the acid nutritional content that comes from stomach [reviewed by [48]]. Although colonization sites of A/E pathogens are different, such studies suggest $HCO_3$- ion is a signalling molecule important to activate virulence determinants required for infection. Finally, it was also determined that ammonium salts and zinc exert an important negative effect on expression and secretion of virulence proteins in EPEC [48, 52-54]. With regard to ammonium, low levels of this ion at ileum allow EPEC infection, whereas high concentrations of this ion found at colon avoid EPEC adherence, and by consequence, expression of virulence factors is turned-off [reviewed by [12]].

### 3.2 Ler and GrlR-GrlA regulatory system: a LEE PAI hierarchical local regulation network.

**Ler, the LEE PAI master regulator.**

*LEE*1-first gene encodes for Ler (Fig 1). Ler proteins of A/E pathogens are highly homologous in structure, function and mechanism of action. Ler exhibits sequence similarity with the H-NS protein family [32]. Similarity between Ler and H-NS is mainly located at the carboxi-terminal domain (where the DNA binding domain resides), but Ler does not work as a transcriptional repressor as members of H-NS family do [55, 56]. Ler is an essential regulator for A/E lesion formation, since it is required to increase transcription of *LEE2, LEE3, LEE4, LEE5* and *grlRA* operons; as well as of *espG*, *escD* and *map* genes, encoded in the LEE PAI [33, 34, 36, 38, 55, 57, 58].

Besides regulating genes at LEE PAI, Ler also increases transcription of genes encoded outside the island such as *espC*, which encodes for an enterotoxin [33]; *lpf* operon encoding for a fimbriae in EHEC [59]; and *stcE* that encodes for a secreted protease exported by the Type 2 secretion system involved in EHEC intimate adhesion to the host cell [33, 60]. Studies on Ler indicate that this protein is a central virulence regulator in A/E pathogens, since it regulates A/E lesion formation and is also required for adhesion to the host cell and toxin production. Therefore, Ler is considered a global regulator of virulence genes in A/E pathogens [33].

Until now, there is no evidence of Ler interacting with the transcription machinery; hence it is not considered a classic transcription activator. Rather, several research groups have proposed that Ler acts as a desrepressor for the

 

transcriptional silencing exerted by global regulator H-NS [33, 36, 55, 57, 58]. Ler does not recognize a DNA consensus sequence, but recognizes AT rich sequences similarly as H-NS do [58].

In fact, studies with EPEC Ler protein indicate it binds the same DNA regions as H-NS, destabilizing DNA-H-NS complexes, alleviating repression and therefore promoting transcription [57, 58]. This H-NS antagonistic function does not apply with all the H-NS repressed genes, since Ler is not able to derepress *proU* or *bgl* operon, demonstrating that Ler works like a specific regulator of virulence genes, instead of a general H-NS antagonist [58]. Given Ler central role in virulence regulation, its expression is a key event in the process of A/E bacterial pathogenicity. Several transcription regulators control *LEE*1 operon expression in EHEC, EPEC and *C. rodentium*. *LEE*1 is positively regulated by IHF, Fis, and GrlA (codified in *grlRA* operon). Particularly, *LEE*1 is also positively regulated by PerC, encoded in EPEC EAF plasmid [34] and Pch, a PerC homologue, codified on EHEC genome [61, 62]. In *C. rodentium*, a PerC homologue has not been reported. In EPEC and EHEC, Ler expression is positively controlled by Quorum sensing, through QseA, which directly activates *LEE*1 transcription [63-65]. A QseA homologue was reported in *C. rodentium*, although a role for this protein has not been demonstrated yet. In EHEC, RsC-RcsD-RcsB system has been reported to activate LEE through Ler regulator [66, 67].

On the other hand, *ler* expression is negatively influenced by proteins such as H-NS and Hha, a protein involved in α-haemolysin regulation in EPEC [68]. Within LEE PAI, another negative regulator has been identified: GrlR that represses *LEE*1 expression [37]. On the other hand, Berdichevsky, *et al*., (2005) reported that Ler represses *LEE*1 transcription in EPEC (negative autoregulation), in order to maintain a just sufficient Ler concentration required for optimal activation of the rest of LEE genes. This observation is controversial since microarray analysis did not show any genes repressed by Ler [reviewed by [56]]. Another microarray analysis reported by Abe et al. (2008), showed negative correlation of several genes with Ler expression, such as genes associated with an acid-resistant phenotype.

**GrlR-GrlA regulatory system.**

The systematic analysis of LEE genes deletion mutants of *C. rodentium* revealed that in addition to Ler, the products of two other genes named *grlR* (orf10) and *grlA* (orf11) were involved in LEE transcriptional regulation [37]. In a mutant lacking GrlA a drastic reduction in EspB and Tir expression and in *LEE*1 transcription was observed, indicating it is a positive regulator. In contrast, the *grlR* mutant caused an increase of *LEE*1 transcription and, conversely when cloned on a multicopy plasmid, GrlR repressed Tir and EspB expression. Such experiments indicated that GrlR acts as a negative modulator. Later, it was demonstrated that *grlR* and *grlA* are organized in an operon. In addition, it was reported that GrlA activates *ler* expression directly, and subsequently, Ler activates the expression of *grlRA* operon, leading to a positive regulatory circuit in *C. rodentium* [38]. On the other hand, two-hybrid experiments revealed that GrlR interacts with itself and with GrlA [69], increasing the complexity of this regulatory network on LEE PAI. GrlR and GrlA are conserved in all the A/E pathogens. Also a GrlR homologue has been identified in *Salmonella bongori*. Furthermore, this gene is located near to a *grlA* homologue [70]. Nevertheless, the role of such genes is unknown. GrlA is 37% and 23% identical with Sgh from Salmonella and CaiF from Enterobacteriaceae, respectively [37].

GrlR crystallographic studies revealed a homodimeric form of β-barrel subunits. A surface-exposed EDED (Glu-Asp-Glu-Asp) motif involved in the interaction with GrlA was also identified [71]. Several studies in EHEC and *C. rodentium* have proposed that GrlR represses LEE gene expression through direct modulation of GrlA activity, by protein-protein interactions [71-73]. Recently, we demonstrated that GrlR negatively regulates *LEE*1, *LEE*2, *LEE*4 and *LEE*5 expression in H-NS absence and independently of its interaction with GrlA. In addition, we found that under inducing growth conditions (DMEM/37°C), GrlA traps GrlR to keep it from repressing LEE gene expression. On the other hand, under repressing growth conditions (LB/37°C), the predominant functional GrlR dimer favours negative control (Lara-Ochoa et al., in preparation). Besides the effect over LEE gene expression, GrlR/GrlA complex is involved on regulation of other pathogenic determinants, such as flagella and enterohaemolysin expression in EHEC [74, 75]. Finally, it was also proposed that ClpXP protease positively controls T3SS expression through direct regulation of GrlR levels, at stationary growth phase [73].

### 3.3 *E. coli* global regulators affecting LEE PAI.

**H-NS silencing.**

One of the most abundant DNA-binding proteins in *E. coli* is H-NS, there are 20,000 copies of it per cell, [reviewed by [76] ]. H-NS is found in *enterobacteriaceae* and related species, playing a role as a histone-like protein, compacting the bacterial chromosome; moreover it silences "foreign" DNA expression by binding to DNA sequences with high A-T content. Due to this characteristic H-NS has become a virulence factor regulator in enteric bacteria [77, 78].

LEE PAI, as several other virulence genes in Gram-negative pathogens, is regulated by H-NS. This protein silences *LEE*1, *LEE*2, *LEE*3, *LEE*4, *LEE*5 and *grlRA* operons [33, 34, 36, 38, 55, 57, 58]. It was reported that in EPEC, H-NS represses *LEE*1 expression at 27$^{o}$C but does not at 37$^{o}$C; moreover, *LEE*2, *LEE*3, *LEE*4, *LEE*5 operons and *espG* are repressed at both temperatures by H-NS. Hence, upon a temperature shift from 27 $^{o}$C to 37 $^{o}$C, Ler is expressed and releases H-NS mediated repression on LEE operons, initiating A/E lesion formation [79]. Biochemical and genetic studies indicate H-NS binds upstream and downstream from silenced promoters, forming a nucleorepressor complex that could trap RNA polymerase. When Ler binds to the promoter upstream region disrupts the repressor complex favouring transcription [57, 58].

**Quorum-sensing system.**

Quorum sensing is a cell to cell-signalling system based on production of compounds known as autoinducers. These compounds allow bacteria to detect their own population as well as population of other bacterial species present in the same environment [80]. This kind of intercellular communication regulates gene expression, allowing that unicellular organisms as *E. coli* can behave as multicellular ones. Virulence, symbiosis, sporulation, biofilm formation and secondary metabolites production are among several important processes regulated by quorum sensing in different bacteria [81].

Sperandio et al. first described quorum-sensing regulation on LEE PAIs in EPEC and EHEC on 1999. LEE transcription is activated by autoinducer-3 (AI3)/epinephrine/norepinephrine, an interspecies signalling molecule present in the intestine [reviewed by[82]]. EPEC and EHEC detect AI-3 through QseC, a sensor histidine kinase, which upon phosphorylation starts a complex signalling cascade to regulate genes involved in A/E lesion and flagella formation [83-85]. QseC activates QseEF, a two-component system, which in turn activates QseA, a LysR family member [86]. QseA directly regulates *LEE*1 transcription [63]. Additionally in EHEC, QseEF activates $espF_u$ transcription, encoding for an effector which is secreted in host cells through the TTSS [86].

Also it was reported that SdiA, a quorum sensing LuxR homologue, represses *espD* and *eae* in EHEC. SdiA is present in EPEC, but the regulatory effect on these genes occurs only in EHEC [87].

A QseA homologue exists in *C. rodentium* but is unknown whether it is involved in *LEE*1 regulation. In this strain, a quorum sensing system has been found, which could be involved on adherence and virulence regulation. However, the genes regulated through this system are yet unknown [88]. Intercellular signalling systems are quite important for A/E pathogens, because the intestine, -where they infect- is highly populated with commensal microflora [56].

**SOS response.**

This system includes more than 40 proteins involved in DNA protection, reparation, replication, mutagenesis and metabolism. SOS response is present in many bacterial species, including *E. coli*, but not in eukaryotic cells. Among the factors turning on the SOS response are: UV radiation, methyl methane sulphonate (MMS), mitomycin C, and some other chemicals altering DNA synthesis, cell division and producing single strand DNA accumulation [89]. There are several reports evidencing that SOS response participates controlling virulence in Gram-negative and Gram-positive pathogens. In EPEC, virulence genes at LEE PAI are regulated through LexA and RecA: *LEE*2 and *LEE*3 transcription increase in presence of mitomycin C and LexA binds to an SOS box located on the *LEE*2-*LEE*3 overlapping promoter region. Also *nleA* transcription, a non-LEE encoded effector but secreted through LEE TTSS, increases in presence of mitomycin C [90].

**Acid-resistance regulators.**

Intestinal pathogens evolved strategies to survive the acid environment present at the stomach in order to arrive at the intestine and develop infection. During this stage, A/E pathogens have to adapt to the pH shift going from the stomach to the intestine, expressing acid resistance genes but repressing virulence genes. EPEC expresses GadX, an activator of genes involved in acid tolerance, such as glutamate decarboxylase, but a repressor of the *perABC* operon located on the EAF plasmid [91]. PerC, encoded in this operon, is a Ler activator, the LEE master regulator [34]; as a result, GadX coordinates properly the acid tolerance and virulence gene expression in the gastrointestinal tract [56].

**Two-component systems.**

Two-component systems are widespread mainly in prokaryotes but absent in animals and humans, representing a potential target in therapeutics; this could be relevant in EHEC infection treatment [reviewed by [86]]. As mentioned, in EHEC the two-component system QseEF, involved in quorum sensing, regulates A/E lesion formation. Also in EHEC two response regulators, YhiF and YhiE, belonging to the LuxR subfamily, were identified as playing a role repressing *LEE*2 and *LEE*4 transcription, hence modulating A/E lesion formation [92].

### 3.4 Regulators acquired by horizontal transfer

**PerC/Pch**

One of the first genetic elements involved in virulence regulation identified in EPEC was *perABC* operon, located in EAF plasmid [93]. This operon consists of 3 genes: *perA* encoding for a protein that belongs to AraC/XylS transcription activators family. PerA activates *perABC* and *bfp* expression (*bfp* encodes for BFP pilus) [34, 53]. The *perB* product has not been characterized in detail but it has been reported that when it is mutated provokes a repression effect on *per* operon [57]. PerC is a Ler transcription activator [57, 93]. Although EAF plasmid is not present in EHEC, five PerC homologues have been identified within prophage regions on EHEC genome, they were designated Pch. Of these, only PchA, PchB and PchC activate LEE gene expression through Ler [61]. *pch* transcription, like *LEE*1 operon, is positively regulated by ppGpp in response to nutrients starvation [66]. The PerC/Pch proteins represent an interesting example of functional coordination between proteins codified in LEE PAI and those codified in different genetic elements.

**EivF and EtrA**

An analysis of EHEC genomic sequence revealed the existence of a group of genes encoding a non-functional T3SS, denominated ETT2 (*E. coli* type III secretion system 2) [45]. This system is very similar to the one encoded in *Salmonella* PAI 1 and it was reported it is present in the majority of *E. coli* strains, independently of their pathogenic

 

features. Although it was shown that ETT2 apparatus carries diverse mutations and is not functional, mutations in the ETT2 encoded regulator genes *eivF* and *etrA* increase EspA, EspB, Tir and EspP secretion. EivF and EtrA repress all LEE operons through *ler* repression, although the mechanism of this regulation is unknown [45]. This is an example of feedback between different T3SS, carried in the cell, in order to avoid superfluous protein expression at the same time and thus prevent an excessive energy cost for the bacterium [45].

**GrvA**

In EHEC, LEE expression is regulated by the RcsC-RcsD-RcsB phosphorelay system through two specific regulator proteins PchA and RcsB [67]. PchA (a *ler* transcriptional activator) is negatively regulated by RcsB, which inhibits *ler* expression and, in consequence inhibits the rest of LEE genes. Positive modulation by this system depends on GrvA, a specific EHEC protein. RcsB positively regulates *grvA* expression and GrvA acts as a positive modulator of *LEE*1 expression [67]. Amino acid sequence analyses indicate that GrvA is probably a transmembranal protein with a DNA binding domain. Until now is known that a great variety of conditions activate this system, nevertheless, little is know about environmental signals involved in activation/repression of these two virulence regulators [67].

**RegA**

In recent years it has been identified RegA, an essential global regulator in *C. rodentium* [46]. RegA (Regulation factor A), has homology to the AraC/XylS transcriptional regulators family. It has been reported that RegA responds to a specific environmental signal of the intestine (bicarbonate ions) to activate genes involved in colonization [50]. Recently, a microarray analysis revealed that RegA activates transcription of 19 genes located in 11 different operons; such genes are involved in intestinal colonization and presumably were acquired by horizontal transfer [50]. From these, the *grlRA* operon codified in LEE PAI, was activated by RegA in presence of bicarbonate. Later, transcriptional fusions analyses confirmed that RegA binds directly to this operon activating transcription [46].

## Concluding Remarks

Intimate adhesion to host cell and A/E lesion formation is a particular characteristic of the A/E pathogens. Intensive research over the last two decades has been devoted to understand the pathogenicity mechanisms used by these organisms. Multidisciplinary approaches from different fields (*e.g.* cell biology, physiology, genomics, molecular biology and bacterial genetics) have converged to unveil how the ample repertoire of A/E virulence factors subvert a wide range of eukaryotic cell processes. A notable feature of A/E pathogens is their ability to transfer its own receptor to the eukaryotic cell establishing an intimate interaction.

In the infected organism A/E lesion is related with diarrhoea induction, becoming the first infection symptom, moreover the underlying mechanisms causing it are poorly understood. Research on this field has concluded there is not a single effector molecule as a causal agent for diarrhoea but the whole set is affecting normal intestinal functioning.

A/E lesion formation requires the expression of LEE PAI genes, present only on A/E pathogens genome. After a bacterium acquires genes encoding for virulence factors by horizontal gene transfer it requires evolve the mechanisms regulating gene expression before to become a pathogen. Particularly *E. coli* regulates LEE gene expression at the transcription level influenced by 1) environmental factors, 2) a LEE-encoded hierarchical regulatory network, 3) a system of global regulators already present in *E. coli*, and 4) a set of regulators horizontally acquired. Besides transcriptional regulation, post-transcriptional and post-translational regulation mechanisms have been evidenced for LEE encoded genes (Fig 2).

It is important to study gene regulation and virulence mechanisms governing pathogenicity on A/E microorganisms in order to develop drugs and therapy to treat and avoid disease caused by these pathogens.

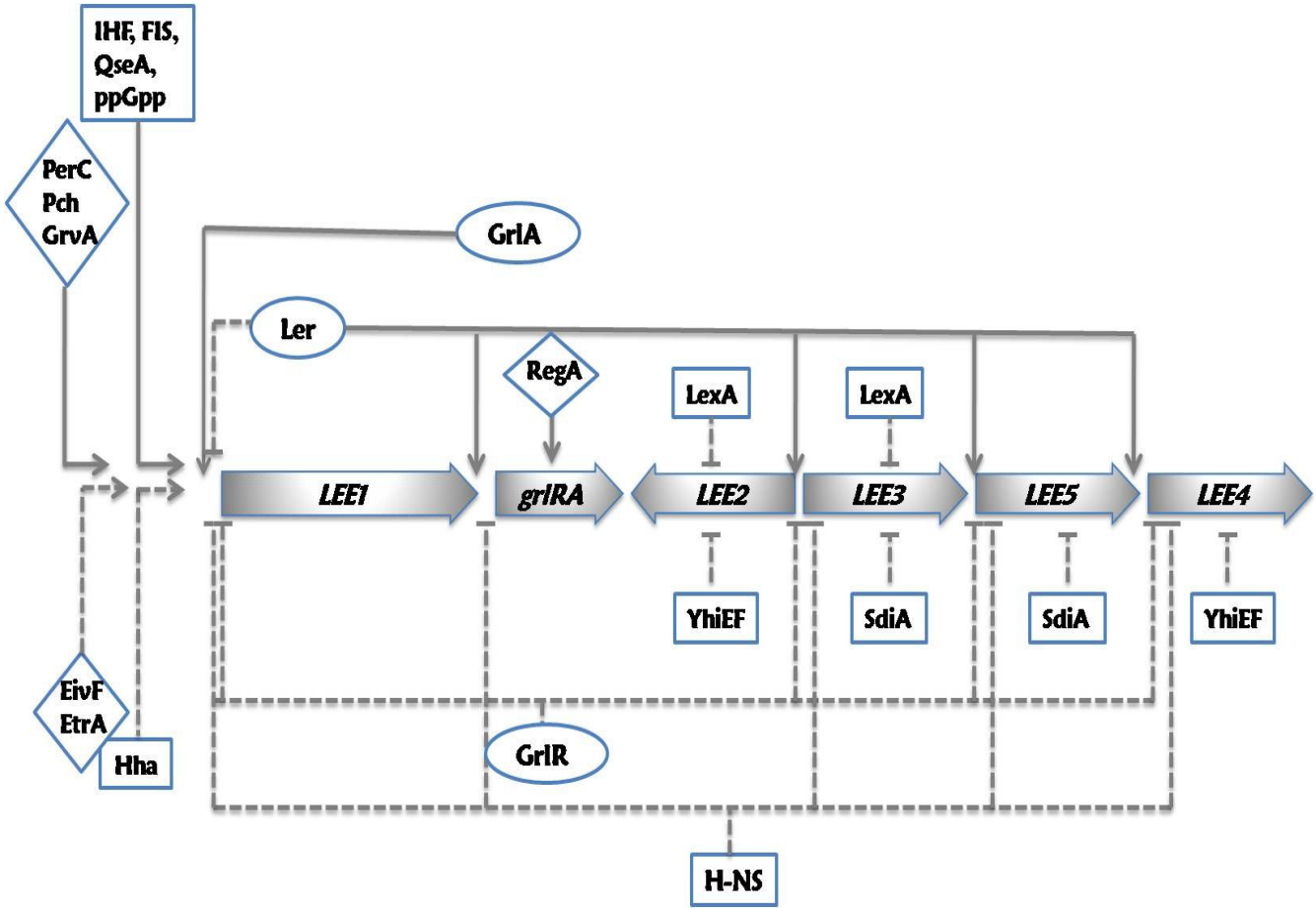


**Fig. 2**. Regulation model of LEE PAI in A/E pathogens. Ler positively regulates LEE gene expression by counteracting H-NS-mediated repression at LEE gene promoters. Ler expression is regulated by specific regulators, such as GrlA, GrlR, and PerC (Pch), as well as by global regulators, such as IHF, Fis, QseA, ppGpp, EtrA, EivF, Hha, RegA, LexA, SdiA and YhiEF.

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