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A Mini-Review of Enteroaggregative *Escherichia coli* with a Specific Target on the Virulence Factors Controlled by the AggR Master Regulator

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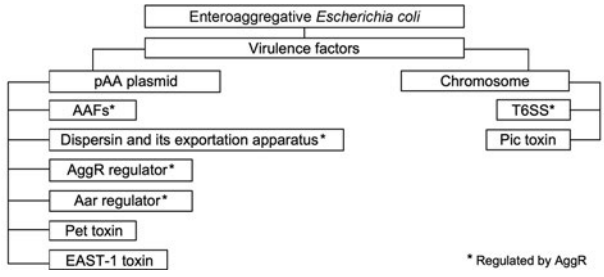
Abstract

Enteroaggregative *Escherichia coli* (EAEC) strains have been linked to several outbreaks of severe diarrhea around the world, and this bacterium is now commonly resistant to antibiotics. As part of the pathophysiology of EAEC, the characteristic pattern of adherence looks like stacked bricks on the intestinal epithelium. This phenotype depends on an aggregative adhesion plasmid (pAA), which codes for a regulatory protein named AggR. The AggR protein is a master regulator that transcriptionally actives the main virulence genes in this *E. coli* pathotype, such as those that encode the aggregative adhesion fimbriae, dispersin and its secretion apparatus, Aar regulatory protein, and type VI secretion system. Several reports have shown that AggR positively affects most EAEC virulence genes, functioning as a classic transcriptional activator in the promoter region of these genes, interacting with the RNA polymerase. This minireview article integrates the information about virulence determinants of EAEC controlled by the AggR regulator.



K e y w o r d s: aggregative adhesion fimbriae, AggR regulator, aggregative adhesion plasmid, dispersin; enteroaggregative *Escherichia coli*

Introduction

Diarrheal diseases are a public health problem affecting children and adults. *Escherichia coli* has been recognized as one of the primary pathogens causing this illness (Gomes et al. 2016). Although this bacterium colonizes the intestine of humans in the first hours of birth and is therefore considered part of the normal microbiota, some strains can become pathogenic (Kaper et al. 2004). *E. coli* strains involved in diarrheal diseases have been divided into six pathogenic types by the recognition of their virulence factors and their pathogenicity mechanisms (Nataro and Kaper 1998). Enteroaggregative *Escherichia coli* (EAEC) is one of these six pathotypes that are linked to diarrhea all over the world. The infections caused by EAEC are characterized by bloody or mucous diarrhea and fever, in addition to other symptoms and signs such as anorexia, borborygmus, tenesmus, nausea, and vomiting (Jenkins 2018). EAEC colonizes the small and large intestines and may slow a child's growth regardless of diarrhea (Rogawski et al. 2017). Subclinical infection occurs mainly in children two years of age (Weintraub 2007); however, it also causes persistent diarrhea in the pediatric population and patients living with the human immunodeficiency virus (HIV) and is a causative agent of traveler's diarrhea (Mayer and Wanke 1995; Kaur et al. 2010). In prospective case-control

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studies performed in Bangladesh and Brazil, EAEC has been linked to malnutrition in children between 6 and 24 months (Lima et al. 2018). In Mexico, EAEC is mainly spread through contaminated salads, desserts, sauces, and contaminated water (Okhuysen and Dupont 2010). The procedure regarded as the gold standard for EAEC detection relies on observing the distinctive "stacked bricks" pattern of adhesion when co-cultured with HEp-2 cells (Flores and Okhuysen 2009). The aggregative adherence (AA) pattern in EAEC is dependent on factors encoded by pAA-borne genes (Vial et al. 1988); it is noteworthy that this pathotype possesses an important transcription regulator of virulence genes named AggR, which is a member of the AraC/XylS family of regulators activating several virulence genes such as the aggregative adherence fimbriae, dispersin and its exportation apparatus, and a type VI secretion system (Morin et al. 2013). This minireview describes the virulence factors controlled by the AggR transcriptional regulator and how it affects the pathogenicity of this *E. coli* diarrheagenic pathotype.

Pathogenesis

EAEC is a diarrhea-causing pathotype that mainly affects children, aging people, and immunocompromised patients in developing and developed countries (Modgil et al. 2020). The EAEC pathogenesis involves three stages: adherence to mucosal epithelial cells, production of toxins, and induction of inflammatory response of the intestinal mucosa. This bacterium adheres to the intestinal epithelial cells in a pattern resembling bricks stacked on each other (Nataro et al. 1987). Its adherence pattern is dependent on organelles called aggregative adherence fimbriae (AAF), and there are at least five different types of them (Nataro et al. 1992; Czeczulin et al. 1997; Boisen et al. 2008; Bernier et al. 2002; Jønsson et al. 2015;). EAEC strains express other adhesive structures such as *E. coli* common pilus (ECP) (Avelino et al. 2010) and type I fimbriae (T1F) (Moreira et al. 2003), and non-canonical adherence factors such as Hra1 (Blanton et al. 2018). EAEC produces different enterotoxins, including the *E. coli* heat-stable enterotoxin (EAST-1) (Savarino et al. 1993), and the plasmid-encoded toxin (Pet) (Eslava et al. 1998), both encoded on the pAA plasmid, as well as the protein involved in colonization (Pic, encoded on the bacterial chromosome), which has the activities of mucinase and mucus secretagogue (Henderson et al. 1999; Flores-Sanchez et al. 2020). Prototypical EAEC strain 042 can induce the release of IL-8, IL-6, and TNF-α from non-polarized Caco-2 or T84 colonic epithelial cells (Steiner et al. 1998; Braga et al. 2018). However, IL-1β and lactoferrin have also been detected in stool samples of traveler's diarrhea (Greenberg et al. 2002). Interestingly, bacterial components such as AAF/II (Harrington et al. 2005) and flagellin (Steiner et al. 2000), encoded in the pAA2 plasmid and chromosome, respectively, showed proinflammatory strong activity.

Virulence factors

Pathogenic strains of *E. coli* have acquired virulence genes obtained from other microorganisms by horizontal gene transfer mechanisms (Kaper et al. 2004). These genes conferred an adaptative advantage to the pathogen, colonizing a new ecological niche by competition with other bacteria and/or damaging host cells. The repertoire of virulence factors ranges from different bacterial components such as fimbriae, secretion systems, enzymes, toxins, and transporters. In the case of EAEC, the most important virulence factors determining its pathogenicity are encoded on a megaplasmid called pAA (Vial et al. 1988). There are also virulence factors encoded on the bacterial chromosome as the type VI secretion system (T6SS) and Pic (Fig. 1). The AggR master regulator, which belongs to the AraC/XylS family, is encoded on the pAA plasmid, controlling the expression of both chromosomal and plasmid genes (Morin et al. 2013). However, there are virulence factors encoded on both plasmid and chromosome, such as Pet/EAST-1 and Pic that AggR does not control. Although EAEC pathogenesis is complex and involves genes not controlled by AggR, the description of the virulence factors regulated by this master regulator is the main focus of this minireview.

Plasmid of Aggregative Adherence (pAA)

Bacterial genomes often contain self-replicating semi-autonomous extrachromosomal DNA components called plasmids that promote the expression of various bacterial phenotypes that impact virulence and antibiotic resistance (Billane et al. 2022). The AA phenotype in EAEC strains is dependent of factors encoded by pAA-borne genes; pAA is a megaplasmid of 72–120 kb (Vial et al. 1988; Jønsson et al. 2017), encoding several virulence factors such as plasmid-encoded toxin (Pet, *pet*), enteroaggregative heat-table toxin (EAST, *astA*), aggregative adherence fimbriae (AAF, *agg/aaf*), anti-aggregation protein (Dispersin, *aap*), anti-aggregation protein transporter (*aatPABCD*) and the gene encoding the AggR transcriptional activator (*aggR*) required for the expression of the most virulence genes (Johnson and Nolan 2009). The "stacked bricks" pattern that characterizes the EAEC adherence on the epithelial cell is a consequence of AAF, which is encoded on the pAA plasmid and promotes biofilm formation for intestinal colonization in the host (Kaper et al. 2004). Moreover, the transcription of the five AAFs variant is activated

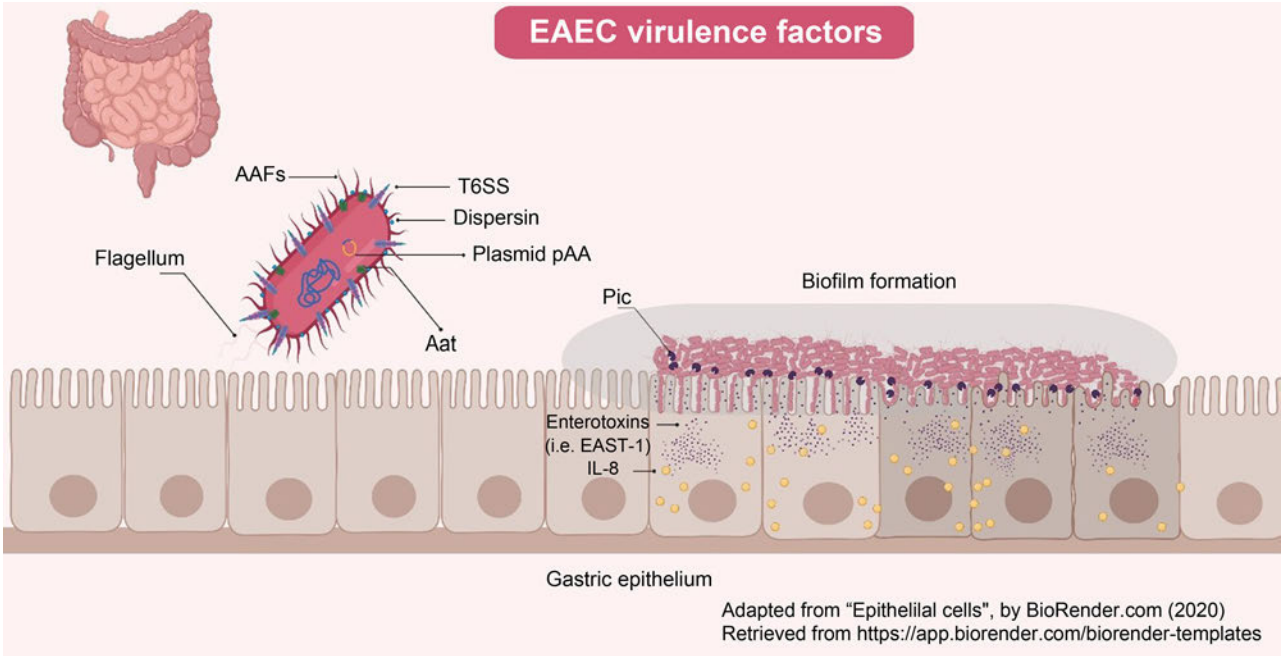

Fig. 1. Schematic representation of EAEC virulence factors.

Most EAEC virulence factors are regulated by the AggR transcriptional activator encoded on the plasmid of aggregative adherence (pAA). EAEC strains adhere in a "brick stacking" pattern and spread in intestinal epithelial cells via aggregative adherence fimbria (AAF). This AAF presents at least five antigenic variants regulated by the AggR master regulator. The AggR transcription factor also activates the genes that encode the anti-aggregation protein (Aap, dispersin) and its anti-aggregation transporter (Aat), and the type VI secretion system (T6SS). EAEC strains can form robust biofilms, which promote high resistance to desiccation, starvation, and host immune response. EAEC secretes at least three toxins: *E. coli* heat-stable enterotoxin (EAST-1), plasmid-encoded toxin, (Pet), and protein involved in colonization (Pic, with mucinase activity), which are not controlled by the AggR regulator, causing an increase in chloride secretion that correlates with secretory diarrhea.

by the AggR master regulator (Nataro et al. 1994; Elias et al. 1999; Boisen et al. 2008; Jønsson et al. 2015), and *aggR*-containing bacteria are named as "typical" EAEC strains (Nataro et al. 1994). The analysis of the sequence of three pAA plasmids of different EAEC strains showed similarity in the content of virulence genes associated with EAEC, but their genomic composition and synteny were significantly different (Johnson and Nolan 2009).

Features and role of AggR as virulence regulator

AggR is the most important transcriptional regulator of EAEC virulence factors (Fig. 2). This protein (29.4 KDa), which is encoded in the pAA plasmid (Nataro et al. 1994), is a member of the AraC-XylS family of transcription factors. AggR shares a high amino acid identity with the enterotoxigenic *E. coli* regulators CfaD (68%), Rns (66%), and CsvR (62%). The *aggR* gene is positively autoregulated by itself, and nucleoid proteins such as Fis and H-NS act as activators and repressors of its expression, respectively (Sheikh et al. 2002; Morin et al. 2010;). While the protein Aar (encoded on the pAA plasmid) negatively affects the *aggR* transcription (Santiago et al. 2014), the (p)ppGpp alarmone (produced by the RelA and SpoT enzymes) has a positive effect on the expression of that regulatory gene (Hüttener et al. 2018). For the reference strain EAEC 042, AggR activates the expression of 44 virulence genes that encode AAF/II (four genes), dispersin (one gene) and its export apparatus (five genes), Aar (one gene), T6SS (16 genes), and proteins with hypothetical and unknown function (17 genes) (Morin et al.

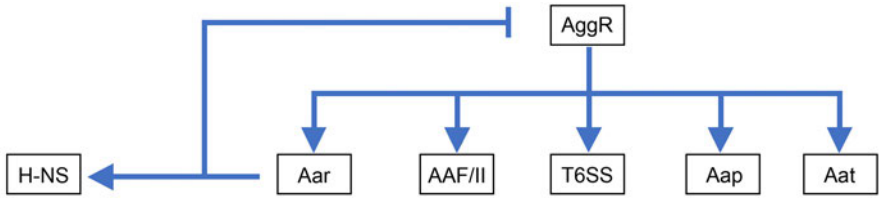

Fig. 2. Regulatory model of AggR and Aar on the EAEC virulence genes.

AggR master regulator activates the virulence regulon that includes: Aar regulatory protein, aggregative adherence fimbriae II (AAF/II), Type VI secretion system (T6SS), anti-aggregation protein (Aap, dispersin), and anti-aggregation transporter (Aat). Aar directly binds AggR, blocking its transcription activity. In addition, Aar forms multiprotein complexes together with H-NS, altering the repressor function of nucleoid protein on its promoter.

2013). Albeit AggR regulon has been reported by two research groups (Morin et al. 2013; Yasir et al. 2019), only the virulence genes under AggR control that were validated by qPCR are depicted in Table I. Interestingly, the role of AggR on the bacterial pathogenesis was evidenced in the EAEC strain O104:H4, which caused the Germany outbreak in 2011: the Δ*aggR* mutant was attenuated in the virulence using the ampicillin-treated mouse model (Boisen et al. 2019). In this sense, hybrid strains such as EAEC/EHEC and EAEC/UPEC possess the *aggR* gene, opening the perspectives about the role of this master regulator in these strains (Nascimento et al. 2021; Kislichkina et al. 2022).

Fimbriae

In more detail, the adhesion of EAEC strains to HEp-2 cells and intestinal mucosa is mediated by AAF, grouped in the DR family of adhesins. These organelles have a semiflexible bundle-forming structure (Nataro 2005). Different AAFs have been observed by electron microscopy so that, depending on the EAEC strain, the main structural subunit of AAF will present five different variants: AggA (AAF/I), AafA (AAF/II), Agg3A (AAF/III), Agg4A (AAF/IV) and Agg5A (AAF/V) (Nataro et al. 1992; Czeczulin et al. 1997; Bernier et al. 2002; Boisen et al. 2008; Jønsson et al. 2015). The AggR transcription factor positively regulates all AAFs variants, and such alleles are also encoded on the pAA plasmid (Nataro et al. 1994; Elias et al. 1999; Boisen et al. 2008; Jønsson et al. 2015). The adhesion molecule is encoded by gene A, which is less similar to the other AAF genes. Genes B-D, however, are similar in all AAF members and make up the auxiliary proteins. The AAF gene cluster has been demonstrated to consist of four genes, from A to D (Elias et al. 1999).

It is known that the AAF/II variant, which expresses the EAEC 042 reference strain, is required to adhere to epithelial cells (Farfan et al. 2008). A study showed that the extracellular matrix glycoprotein fibronectin acts as a cellular receptor and is involved in how these fimbriae cause EAEC to stick to the surface of cells but not in the inflammatory response (Farfan et al. 2008; Yáñez et al. 2016). Regarding virulence, the absence of AggA fimbria in the EAEC strain O104:H4, presented a diminishing in the intestinal colonization in the ampicillin-treated mouse model (Boisen et al. 2019).

Dispersin and its secretion apparatus

Dispersin is a small protein (10.2 kDa) encoded by the *aap* gene, which is located upstream of the *aggR* regulatory gene and activated by the AggR regulator (Sheikh et al. 2002). The primary function of dispersin is to form a surface layer that disperses bacteria (Sheikh et al. 2002). Due to its location on the bacterial surface, dispersin is highly immunogenic (Nataro et al. 1995; Huang et al. 2008). Dispersin attaches non-covalently to

Table I
Virulence genes regulated by AggR*.

Location	Virulence factor	Regulated genes	Description
pAA2 plasmid	AAF/II	*aafA*, *aafB*, *aafC*, *aafD*	major subunit, minor subunit, usher protein, chaperone of AAF/II
	dispersin	*aap*	dispersin (antiaggregation protein)
	dispersin export apparatus	*aatA*, *aatB*, *aatC*, *aatD*, *aatP*	TolC-like outer membrane protein, hypothetic protein, ABC transporter, N-acyltransferase, permease protein
	AggR	*aggR*	AraC/XylS-type transcriptional regulator
	Aar	*aar*	ANR-family regulator
	ORF3	EC042_RS26240 (pAA003)	isoprenyl diphosphate synthase
	Idi (ORF4)	*idi*	isopentenyl-diphosphate delta-isomerase
	Shf	*shf*	polysaccharide deacetylase
		EC042_RS30880 (pAA005A), EC042_RS26330 (pAA0020), (pAA0047), (pAA0056)	hypothetical proteins
Chromosome	Type VI secretion system	*aaiA-U* (EC042_RS24360–EC042_RS24465)	T6SS putative proteins
		EC042_RS16995 (EC042_3181)	putative transcriptional regulator
		EC042_RS17000 (EC042_3182)	ParB-type nuclease
		EC042_RS17030 (EC042_3187)	putative helicase
		EC042_RS17005, EC042_RS17010, EC042_RS21290 (EC042_3183, EC042_3184, EC042_4006)	hypothetic proteins

* – only virulence genes under AggR regulation validated by RT-qPCR are presented

the outer membrane and lipopolysaccharide by electrostatic interactions (Velarde et al. 2007). This attraction would antagonize the extended binding from AAF to the surface of the bacteria, partially affecting the interaction with any component from the mucosal surface (Velarde et al. 2007). However, it was reported that dispersin is palmitoylated at the N-terminal, permitting its attachment to the membrane by this fatty acid (Belmont-Monroy et al. 2020). In addition, the role of dispersin in the attachment between bacteria is important for the resistance to ciprofloxacin since the minimum inhibitory concentration of this antibiotic is higher in the Δ*aap* mutant *vs.* the wild-type strain (Mortensen et al. 2011). However, Blanton et al. (2018) reported that the increase in both aggregative adherence and intestinal colonization observed in the absence of dispersin, is mediated by the Hra1 non-structural adhesin and not for AAF/II. Dispersin can bind to extracellular matrix proteins such as laminin and plasminogen, producing plasmin with the participation of plasminogen activator, suggesting a role of this protein in the pathogenicity mechanisms related to a systemic infection like the bacteremia (Moraes et al. 2020).

The transport of dispersin to the bacterial surface requires of an ABC transporter complex (Aat), which comprises five genes (*aatPABCD*) encoded on the pAA plasmid, and it is also positively regulated by AggR (Nishi et al. 2003). The *aatPABCD* locus encodes permease protein, N-acyltransferase, ABC transporter, hypothetic protein, and TolC-like outer membrane protein, respectively (Nishi et al. 2003; Iwashita et al. 2006; Belmont-Monroy et al. 2020). The transport of dispersin involves the AatA channel, which requires the AatC ATPase activity. While AatB could act as an adaptor protein, AatD is a lipoprotein that acylates to the dispersin, and this acyltransferase activity is required for the EAEC's virulence using the streptomycin-treated mouse model (Belmont-Monroy et al. 2020). The *aap*/*attPABCD* genes are prevalent in EAEC strains, and *cexE* homologues have been found in non-pathogenic *E. coli* and diarrheagenic pathotypes such as enterotoxigenic *E. coli*, and in *Yersinia enterocolitica*, *Providencia alcalifaciens*, and *Citrobacter rodentium* (Monteiro et al. 2009; Rivas et al. 2020).

Aar regulatory protein

In addition to its auto-regulatory activity, AggR positively controls the *aar* gene, which encodes Aar regulatory protein (Santiago et al. 2014). This small protein belongs to the ANR (AraC Negative Regulator) family and acts as a negative regulator of virulence factors controlled by AggR (Santiago et al. 2014), showing two different activities (Fig. 2): (i) interacts with AggR blocking its activity (Santiago et al. 2016), (ii) interacts with H-NS stabilizing or inhibiting the formation of multiprotein complexes (Santiago et al. 2017). This dual function depends on its concentration: Aar binds H-NS at low protein levels, resulting in a derepression of genes silenced by the nucleoid protein. When the Aar concentration is high, this small protein also binds to AggR, diminishing the positive effect of the master regulator on their target genes (Mickey and Nataro 2020).

T6SS and hypothetical proteins

The T6SS is a nanomachine found mainly in Proteobacteria and Bacteroidetes with multiple functions such as bacterial competition, biofilm formation, cell adherence, and invasion (Coyne et al. 2016; Coyne and Comstock 2019). Two T6SSs have been characterized in EAEC using strain 17–2: T6SS-1 and T6SS-3 (Journet and Cascales 2016). T6SS-3, which encompasses the *aaiA-U* cluster, is required for the bacterial competition since the AaiC protein is a homolog to Hcp, forming the inner tube of T6SS, and proteins such as AaiB, AaiG, AaiO, and AaiP are required for the secretion of AaiC. AggR regulator activates the T6SS-3 genes in conditions mimicking the intracellular environment (Dudley et al. 2006). AggR would directly regulate the *aaiA-U* cluster; however, DNA-protein binding experiments are necessary to demonstrate the direct role of AggR on these genes.

Other proteins encoded in the pAA2 megaplasmid are beginning to be characterized. The product of pAA003 and *idi* (pAA004) genes show homology for isoprenyl diphosphate synthase and isopentenyl-diphosphate delta-isomerase, respectively (Morin et al. 2013). Single and double mutants in both genes showed a lower viability in the presence of defensin-1 and polymyxin, compared to the wild-type strain, suggesting that both proteins are required to counteract the effect of antimicrobial cationic peptides affecting the cellular envelope (Morin et al. 2013). The definition of the role of other putative proteins belonging to AggR regulon will elucidate the myriad of virulence factors that use EAEC during its pathogenesis.

Biofilm

One of the most critical virulence phenotypes in EAEC is biofilm formation, primarily observed in the colon (Al Safadi et al. 2012; Boisen et al. 2019; Petro et al. 2020). The development of biofilms has been linked to clinical isolates that have the *aggR* regulatory gene with other virulence genes such as *aatA* (dispersin transporter), *irp2* (yersiniabactin biosynthesis gene), and *set1A* (*Shigella* enterotoxin-1) (Mohamed et al. 2007; Pereira et al. 2010; Petro et al. 2020). Extracytoplasmic proteins such as Agn43 and YafK are required for the EAEC biofilm formation (Sheikh et al. 2001; Schiebel et al. 2017). However, by the generation of null

mutants and complementation assays, the biogenesis of the AAFs variants has been described as crucial for the biofilm formation in the EAEC strains (Sheikh et al. 2002; Boisen et al. 2008; Jønsson et al. 2015; Nagy et al. 2016;). Therefore, any regulatory component (direct or indirect) that affects such biogenesis will impact the biofilm formation. In this sense, Fis nucleoid protein and the (p)ppGpp alarmone positively affect the *aggR* transcription and, subsequently, biofilm formation in strain 042, since this phenotype is AAF/II-dependent (Sheikh et al. 2001; Hüttener et al. 2018).

Conclusion

Due to its ability to cause deadly diarrhea in children, aging, and immunocompromised populations, EAEC is currently regarded as one of the most epidemiologically significant enteropathogens in developing countries. The EAEC adherence pattern depends on the expression of AAFs, which are encoded on the pAA megaplasmid and are also required for biofilm formation. Canonic virulence factors such as dispersin show additional functions not restricted to its anti-aggregation function. At the molecular level, AggR is the master regulator that positively controls the most EAEC virulence factors. At the top of the regulatory cascade, AggR positively regulates virulence genes encoded in both plasmid and chromosome. In this sense, the AggR-mediated activation of T6SS promotes the bacterial competition of this pathotype with other bacteria present in the gut. New virulence genes belonging to the AggR regulon have been recently identified and characterized, opening the perspective about the pathogenicity mechanisms that use EAEC to colonize the host successfully. Therefore, the regulation of EAEC pathogenesis is based on controlling the AggR levels both at the transcriptional (H-NS and AggR itself) and post-translational (Aar) levels. Hence, determining the AggR levels during the infection and how they differentially affect both the expression of the virulence factors and the interaction between them is one of the main goals for better understanding complex mechanisms that use EAEC to colonize the intestine tract.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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