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The role of quorum sensing in *Escherichia coli* (ETEC) virulence factors

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ABSTRACT

Quorum sensing (QS) is a signaling system among bacteria mediated by auto-inducer substances (AI). Whenever the concentration of these molecules reaches a threshold corresponding to a high cell density or quorum, the whole population starts a coordinated expression of specific genes. Studies have shown that epinephrine is also responsible for activating specific bacterial genes. This work aimed to investigate the role of conditioned medium (containing AI), epinephrine and their association on growth, motility, F4 fimbriae and heat-labile toxin (LT) expression on enterotoxigenic *Escherichia coli* (ETEC, E68). A significant increase in motility, F4 and LT expression, was observed in the ETEC culture supplemented with conditioned medium and epinephrine. These findings suggest that ETEC uses some components of conditioned medium (e.g., AI molecules), host molecules (epinephrine), and their association to modulate the expression of important virulence genes.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains are a major cause of diarrheal disease in humans and animals (Zhu et al., 2011). ETEC is the most common cause of traveler's diarrhea and can be fatal for children (Qadri et al., 2005). The major virulence factors are bacterial fimbriae that mediate bacterial attachment to host enterocytes allowing its colonization and proliferation, and the production of enterotoxins, which stimulate fluid and electrolyte secretion by intestinal cells leading to diarrhea (Fairbrother et al., 2005).

ETEC in pigs is responsible for much of the neonatal, and a majority of the post weaning diarrheal infections (Berberov et al., 2004). Stress caused by handling, isolation of litters from the sow, or by cold, has been reported to increase fecal excretion of ETEC in comparison to control piglets (Dowd et al., 2007). Stress induces the activation of the adrenal, leading to the release of stress-related catecholamine hormones that can impair immune response and activate growth and expression of virulence factors in bacteria (Jones et al., 2001). The catecholamines (epinephrine and norepinephrine) can be present in the gastrointestinal tract (Horger et al., 1998). Norepinephrine is produced within adrenergic neurons present in the enteric nervous system, and epinephrine is synthesized in both the central nervous system and the adrenal medulla and is involved in systemic responses (Purves et al., 2001). The levels of epinephrine in the intestine are in the micromolar range, similar to the level tested in the present study, however, during an ETEC infection, the altered electrolyte secretion may compromise the integrity of the enterocyte, leading to an increase in its concentration (Polotsky et al., 1994; Freestone et al., 2008).

E. coli strains regulate their virulence gene expression in response to a variety of environmental factors and can use quorum sensing (QS) to modulate gene expression. QS is a bacterial cell-to-cell communication mechanism, comprising the production and detection of autoinducers (AI). Report of a signaling molecule, AI-3, whose synthesis is not dependent on LuxS (Sperandio et al., 2003), suggests that this molecule is involved in Enterohemorrhagic *E. coli* (EHEC) cross talk with the epinephrine-norepinephrine host signaling system (Freestone et al., 2002). EHEC senses the host

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hormones epinephrine and norepinephrine through the membrane protein QseC (Walters et al., 2006). QseC senses both AI-3 and epinephrine and thus functions in interkingdom cross-signaling (Rasko et al., 2008; Moreira et al., 2010). QseC is part of a two-component system, QseB/C, in which QseC phosphorylate on binding of its ligand and transfer the phosphate moiety to QseB transcriptional regulators, by doing so modulating the expression of genes under their control (Sperandio et al., 2002). However, two-component sensory systems are known to integrate multiple signals and the extent to which such sensors influence virulence by sensing stress-related catecholamines is not totally understood.

Given that AI-3 production is not limited to EHEC strains, and that AI-3/epinephrine may be involved in interspecies/interkingdom signaling, one can suggest that this signaling pathway might also play a role in the pathogenesis of ETEC. The specific aim of this study was to determine the effect of conditioned medium (containing factors e.g., autoinducer molecules), and epinephrine in the growth, motility, fimbriae and heat-labile toxin gene expression in ETEC E68.

2. Material and methods

2.1. Bacteria and culture media

The *E. coli* E68 (O141; K88ab; H4) (Gil Turnes et al., 1999), and *E. coli* DH5α (*fhuA2 lac(del)U169 phoA glnV44 Φ80′ lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*, Invitrogen) strains used in this experiment were provided by bacteria collection of Cenbiot Bacteriology Laboratory – UFPel. The culture media used was BrainHeart Infusion broth (BHI, Difco), Luria Bertani (LB, Acumedia), and Minimum Essential Medium eagle (MEM, Sigma). Supplements of epinephrine (Hipolabor), and atenolol a β adrenergic receptor-blocker (Farmanostra) were also used (Sperandio et al., 2003).

2.2. Conditioned medium preparation

Conditioned medium refers to the filtered *E. coli* culture supernatant (containing autoinducers). For the preparation of conditioned medium, ETEC was grown in 500 ml flasks with Luria Bertani medium corresponding to 10% of their volume, incubated at 37 °C for 7 h in an orbital shaker (CERTOMAT® BS-T) at 150 rpm. Afterwards, the culture was centrifuged at 10,000 × *g* for 20 min, the supernatant was filtered (0.22 μm filter - Millipore), and the filtrate was used to condition the media (Sperandio et al., 2002). The same procedure was done for the *E. coli* DH5α which does not produce AI-2 and AI-3, or produces at insignificant and biologically inactive quantities.

2.3. Cell growth dynamics

To evaluate the influence of the conditioned medium on cellular growth, ETEC was grown in LB to obtain a pre-inoculum and then adjusted to OD 1.0 (A_{600}); 2 ml of the inoculum (1×10^9 CFU/ml) for each treatment analyzed. The treatments corresponded to the addition of conditioned medium and epinephrine to cultures in LB broth. These treatments were: control (LB medium); conditioned medium 50%, 50 μM epinephrine, conditioned medium 50% + 50 μM epinephrine, 200 μM β adrenergic blocker, atenolol (to evaluate the role of AI-3/cathecolamine signaling pathway), and 50% DH5α conditioned medium. The different treatment cultures were incubated at 37 °C in an orbital shaker for 7 h at 130 rpm. Aliquot were taken hourly from each treatment and quantified by optical density with a spectrophotometer (Biospectro SP-22).

2.4. Motility

The motility of ETEC was evaluated by agar assay, and qPCR. The motility agar assay was performed according to Sperandio et al. (2002) with some modifications. These modifications were related to the culture medium, treatments and incubation period. Briefly, ETEC was grown in MEM, incubated at 37 °C for 7 h at 130 rpm, in an orbital shaker. From this culture (OD = 0.8), 1 μl (6.5×10^5 CFU/ml) was inoculated into a Petri dish containing MEM solidified with 0.3% agar. The treatments were: control (LB medium); conditioned medium 50%, 50 μM epinephrine, conditioned medium 50% + 50 μM epinephrine, atenolol (200 μM), and 50% DH5α conditioned medium. The plates were incubated at 37 °C for 12 h, and the motility halos were measured with a caliper.

2.5. Fimbriae expression

The fimbriae expression by ETEC was evaluated by hemagglutination, ELISA, and qPCR for each of the treatments described above. For hemagglutination, an adapted version of the hemagglutination (HA) technique proposed by Hovelius and Mardh (1979) was used. Briefly, the reaction consisted of joining cultures (adjusted to an OD = 1.0) diluted in saline (500 μl/well), and 500 μl of a 0.5% suspension of chicken erythrocytes in saline to eighty-well U-bottom plates. The plates were then incubated at 4 °C for 2 h for later reading. ELISA was performed with the ETEC cultures of the different treatments, which were formalin inactivated and then used as antigens. The plates (Nunc) were sensitized with *E. coli* cultures (50 μl/well, 1×10^8 CFU/ml) in carbonate-bicarbonate buffer (pH 9.6) for 1 h at 37 °C. Rabbit anti-F4 serum, kindly provided by Professor Dr. Carlos Gil Turnes (UFPel), (50 μl/well) diluted at 1:50 in PBS-T (phosphate buffer containing 0.5% Tween 20) was added to the plates, which were then incubated for 1 h at 37 °C. Anti-rabbit immunoglobulin peroxidase conjugate (DAKO-PATTS A/S) diluted at 1:2000 in PBS-T was used as secondary antibody, and incubated at 37 °C for 1 h. Next, the plates were washed 5 times in PBS-T, and 50 μl *ortho*-23 phenylenediamine (OPD) substrate/chromagen was added and allowed to react for 15 min in the dark, at room temperature. Absorbencies were measured in a micro plate reader (MR 700 microplate reader) at 450 nm.

To measure gene expression, ETEC was grown in LB at 37 °C for 7 h at 130 rpm in an orbital shaker. Following this incubation period, the culture was centrifuged at 13,000 × *g* for 10 min. First, we analyzed the treatments at 2 h of culture, then, to study the expression dynamics, samples were collected every 30 min, totaling four collections, and only from the conditioned medium 50% + 50 μM epinephrine treatment. These time points were chosen because they corresponded to the biggest differences found in pilot experiments. After incubation, samples were pelleted and suspended in TRIzol (Invitrogen) and kept at −70 °C. RNA was extracted according to the manufacturer's instructions, quantified in a spectrophotometer (Nanovue) and standardized at 1 ng for each cDNA synthesis reaction. cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the

Table 1
Oligonucleotides used for quantitative real-time polymerase chain reaction (qPCR).

Gene	Forward 5′-3′	Reverse 5′-3′
faeG	CGCAGGTTCTTACAGGGAAA	GACCGTTTGCAATACCCAGT
faeC	CTGTTGATGCACAGGCTGAT	TACTCTCCCGCCTTCACATC
eltB	ACGGAGCTCCCCAGA CTAT	AATGTTTCGCCGCTCTTAAA
fliC	TCGACAAATTCCGCTCCTC	GGTTGGTGGTGGTGTTGTTC
rpoA	GCGCTCATCTTCTTCCGAAT	CGCGGTCGTGGTTATGTG

manufacturer's instructions, quantified in a spectrophotometer (Nanovue) and then stored at −20 °C until use. Gene expression was determined by quantitative real-time polymerase chain reaction (qPCR). The primers used in this experiment were designed using the program PRIMER 3 v. 0.4.0 (Table 1) following the GenBank access number AY437806 for *faeG*; AF450247 for *faeC*; M17873.1 for *eltB*; AY906923.1 for *fliC*. Reactions were performed in duplicate using a Platinum SYBR Green qPCR SuperMix UDG Kit (Applied Biosystems) according to manufacturer's instructions. The real-time PCR reaction consisted of 200 ng of cDNA, 6.25 μl of Platinum SYBR Green, 0.25 μl of Rox Reference Dye, 0.5 μl of each primer and water up to a 12.5 μl volume. The samples underwent 45 cycles with the following conditions: 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. The analysis of the resulting data was performed using the real-time MxPro–Mx3005P software. The relative amount of mRNA for each gene was determined by the comparative threshold cycle ($\Delta\Delta C_T$) method, which was standardized using the *rpoA* RNA gene sequence (Anonymous, 1997).

2.6. Statistical analysis

Data was analyzed by a *t* test using the 2009 version of the Statistics software (SAS®, 2009), for hemagglutination experiments, we used the Chi square test, *p*-values of <0.05 were considered significant. Each assay was performed at least in three separate experiments.

3. Results

3.1. Cell growth dynamics

The addition of conditioned medium to the cultures increased ETEC E68 growth significantly ($p < 0.05$) starting at the 3rd hour of culture and continuing at the 4th and 5th hour, however by the 6th hour the difference was reduced and by the 7th hour no difference among the control and other treatments were observed (Fig. 1). Epinephrine alone at concentrations of 50 μM did not have any effect on ETEC growth dynamics, and when combined with the conditioned medium the growth curve was similar to the conditioned medium alone (Fig. 1). The addition of atenolol to the cultures with conditioned medium and with conditioned medium 50% + 50 μM epinephrine inhibited its effect, bringing the dynamics to the control level (Fig. 1). The conditioned medium prepared from the cultures of DH5α did not show any effect on the growth dynamic.

3.2. Motility

Using conditioned medium, an increase in the halo diameter was observed. Epinephrine at 50 μM did not show any effect on motility, however when combined with conditioned medium it was able to increase the motility significantly ($p < 0.05$) up to ~7.5 fold (Fig. 2). The addition of atenolol to the treatments was able to inhibit the motility increase (halo diameter), and the conditioned medium from DH5α did not show any effect on motility (Fig. 2).

3.3. Fimbriae expression

When examining the expression of F4 by ETEC E68 hemagglutination, we found that the treatment with the combination of conditioned medium and epinephrine was able to modulate its expression. The highest hemagglutination titer, 64 hemagglutination units, was observed in the conditioned medium 50% + 50 μM epinephrine treatment; this was significantly increased as compared to other treatments ($p < 0.05$) (Fig. 3A). ELISA also revealed that the treatment of conditioned medium 50% + 50 μM epinephrine significantly increased fimbriae

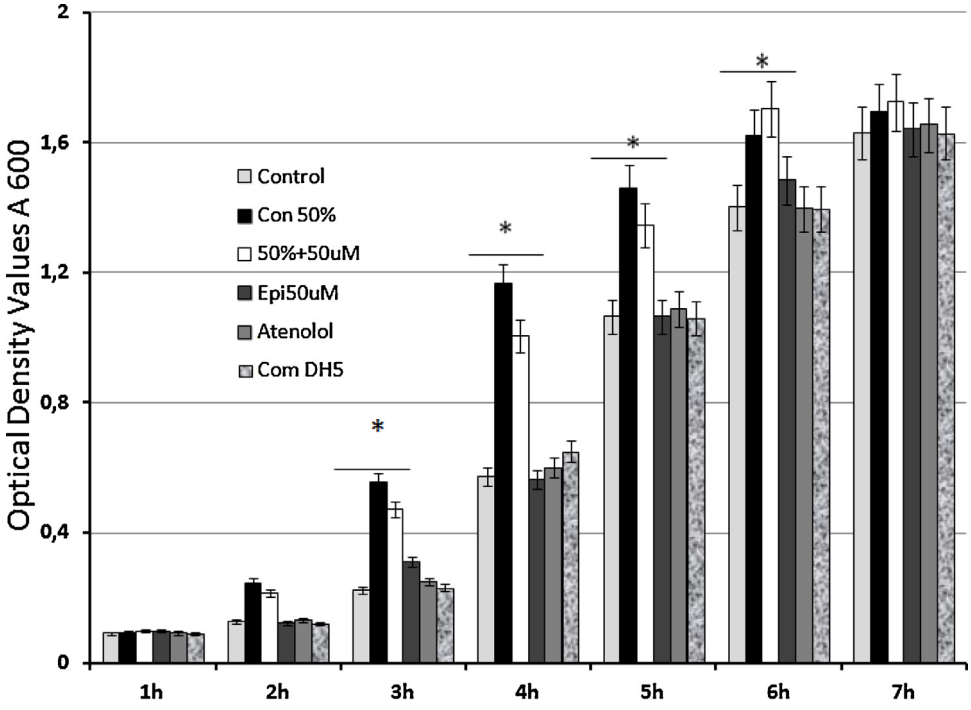

Fig. 1. ETEC growth dynamic. The data represents the mean optical density of growth curve of ETEC in Luria Bertani, LB (Control), LB combined with 50% conditioned medium (Con 50%), LB combined with 50% conditioned medium + epinephrine 50 μM (50%+ 50 μM), LB combined with epinephrine 50 μM (Epi50 μM), LB combined with 50% conditioned medium combined with epinephrine 50 μM with atenolol (200 μM) (Atenolol), and LB combined with 50% conditioned medium from *E. coli* DH5α (Con DH5). The data represent the mean of three independent experiments (±standard deviation), and asterisks (*) means a significant ($p < 0.05$) difference between the control and treatment.

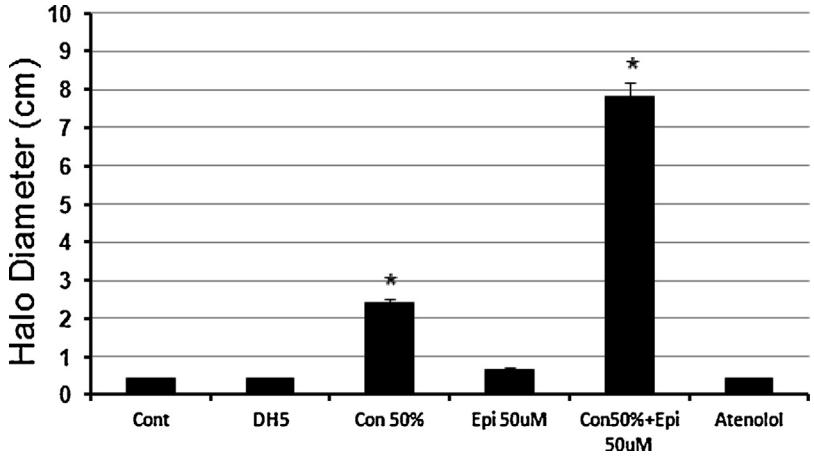

Fig. 2. ETEC motility. The data represent the mean halo diameter in MEM plus 0.3% agar after treating with: Luria Bertani, LB (Cont), LB combined with 50% conditioned medium from *E. coli* DH5α (DH5), LB combined with 50% conditioned media (Con 50%), LB combined with epinephrine 50 μM (Epi 50 μM), LB combined with 50% conditioned medium (Con 50%) and epinephrine 50 μM (Con50%+ Epi50 μM), LB combined with 50% conditioned medium combined with epinephrine 50 μM with atenolol (200 μM) (Atenolol). The data represent the mean of three independent experiments (±standard deviation), and asterisks (*) means a significant ($p < 0.05$) difference when comparing to the control group.

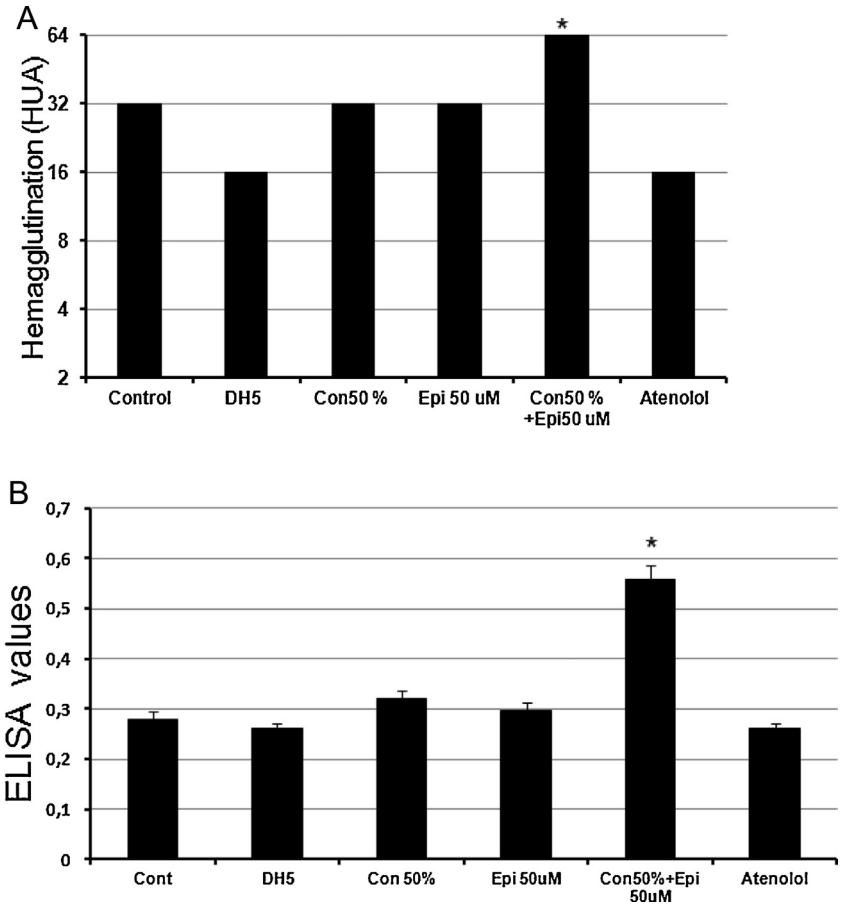

Fig. 3. F4 fimbriae expression. (A) Data represents hemagglutination titer from ETEC cultured in Luria Bertani (LB) media after treat with: Luria Bertani, LB (Cont), LB combined with 50% conditioned medium from *E. coli* DHα (DH5), LB combined with 50% conditioned medium (Con 50%), LB combined with epinephrine 50 μM (Epi50 μM), LB combined with 50% conditioned medium (Con 50%) and epinephrine 50 μM (Con50%+ Epi50 μM), LB combined with 50% conditioned medium combined with epinephrine 50 μM with atenolol (200 μM) (Atenolol). (B) F4 ELISA with anti-F4 antibody. The data represents ETEC cultured in Luria Bertani, LB, (Cont), LB combined with 50% conditioned medium from *E. coli* DH5α (DH5), LB combined with 50% conditioned medium (Con 50%), LB combined with epinephrine 50 μM (Epi50 μM), LB combined with 50% conditioned medium (Con 50%) and epinephrine 50 μM (Con50%+ Epi50 μM), LB combined with 50% conditioned medium combined with epinephrine 50 μM with atenolol (200 μM) (Atenolol). The data represent the mean of three independent experiments (±standard deviation), and asterisks (*) means a significant ($p < 0.05$) difference when comparing to the control group.

expression as compared with other treatments ($p < 0.05$) (Fig. 3B), corroborating with the hemagglutination findings. In addition, we observed in both assays, that atenolol was able to block the treatment effect, and the DH5α conditioned medium had no effect (Fig. 3).

3.4. Gene expression

First, we observed that at the 2nd hour of culture, the only treatment capable to modulate a significant up regulation on *fli*C, *fae*G, *fae*C, and *elt*B of ETEC E68 was the conditioned medium 50% + 50 μM epinephrine (Fig. 4A). Next, we found a time-dependent modulation on gene expression in the treatment group with conditioned medium and 50 μM epinephrine, where at 30 min *elt*B, *fae*C and *fae*G were up regulated compared with controls, and the highest gene expression occurred at 2 h of culture for all gene studied. The gene expression dynamics were as follows: for flagella, the *fliC* expression increased 0.4, 6.2 and 11 fold, at 30 min, 1 h and 2 h respectively. For F4 fimbriae the *fae*G expression increased 1.4, 6.5 and 9.3, respectively, and for *fae*C we observed an expression increase of 1.8, 4.4 and 8.4 at 30 min, 1 h and 2 h, respectively. The heat-labile *elt*B gene demonstrated an increased expression of 3.0, 3.6 and 11 fold at 30 min, 1 h and 2 h, respectively (Fig. 4B). The addition of atenolol to the treatment was able to significantly ($p < 0.05$) reduce the expression of all genes tested, suggesting that the signaling might be through AI-3/catecholamine pathway (Fig. 4A).

4. Discussion

Studies have shown that the use of catecholamines in culture media promotes the multiplication, motility and expression of genes involved in bacterial virulence (Toscano et al., 2007; Bearson and Bearson, 2008; Bearson et al., 2010). The results presented in this study suggest that ETEC E68 may utilize epinephrine, as well as its own molecules (conditioned medium, e.g., AI-3) as an environmental cue to modulate virulence factors essential for its pathogenesis. We have found that the combination of conditioned

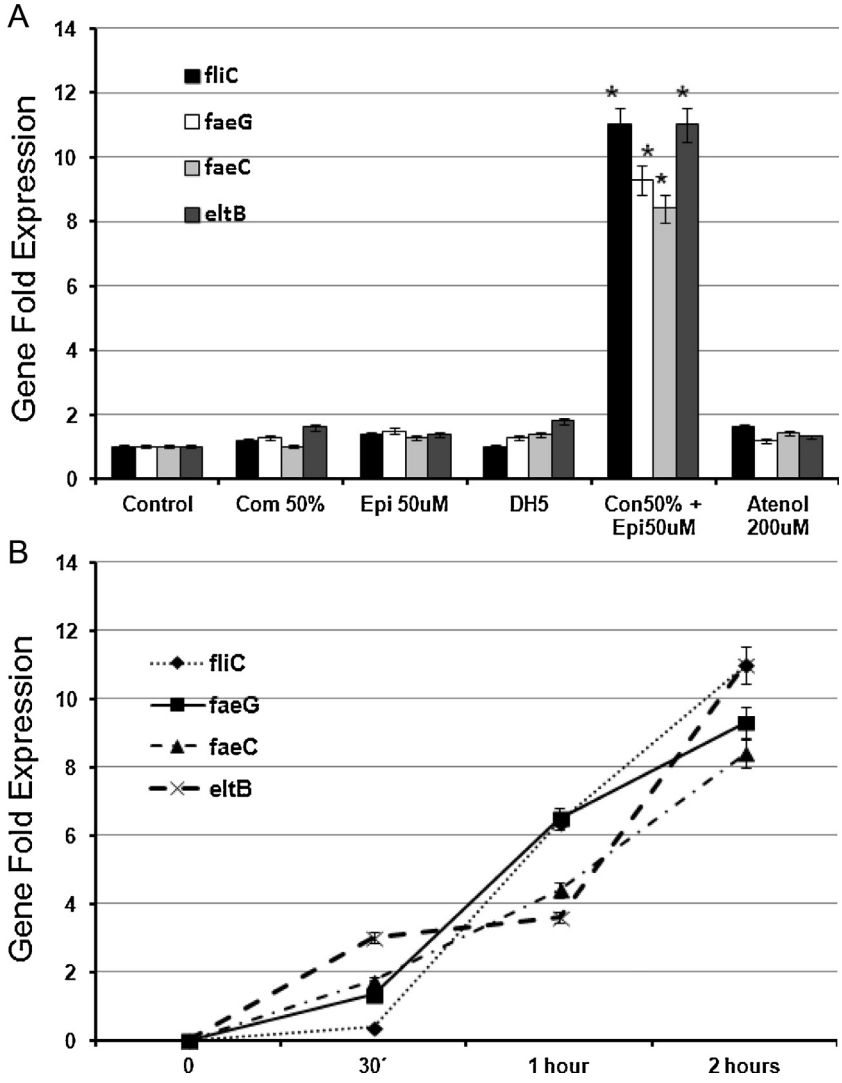

Fig. 4. qPCR expression for ETEC *fliC*,*fae*G, *fae*C and *elt*B genes. (A) The data represents fold increase gene expression (ΔDC_T) in ETEC cultured in Luria Bertani, LB (Control), LB combined with 50% conditioned medium (Con 50%), LB combined with epinephrine 50 μM (Epi50 μM), LB combined with 50% conditioned medium from *E. coli* DH5α (DH5), LB combined with 50% conditioned medium (Con 50%) and epinephrine 50 μM (Con50%+ Epi50 μM), LB combined with 50% conditioned medium combined with epinephrine 50 μM with atenolol (200 μM) (Atenolol). (B) Gene expression dynamics of *fliC*, *fae*G, *fae*C and *elt*B following Con50%+ Epi50 μM treatment. The data represent the mean of three independent experiments (±standard deviation), and asterisks (*) means a significant ($p < 0.05$) difference when comparing to the control group.

medium with epinephrine increases the in vitro motility, and expression of the ETEC E68 F4 fimbriae as well as the LT toxin. Given the presence of stress-related hormones in the host, these findings suggest that in the in vivo circumstance, epinephrine and autoinducers play a role in the pathogenesis of ETEC E68 infection. However, further studies using better AI characterization as well different ETEC strains need to be done, since the regulatory circuit observed in this study may be unique to ETEC E68.

In this study the presence of autoinducers was established by the use of conditioned medium; however we were not able to demonstrate the presence of any specific autoinducer (e.g., AI-2, AI-3, and indole). On the other hand, it is worth mentioning that even without autoinducer characterization in the conditioned medium, one can infer that most of the effect observed was due to AI-3, since its effect could be inhibited by the addition of β adrenergic-receptor blocker (atenolol) to the treatments (Sperandio et al., 2003; Clarke and Sperandio, 2005). Also, using as control of possible other metabolic components, the conditioned medium from *E. coli* DH5α which does not produce AI-2 and AI-3 (or produces at insignificant and biologically inactive quantities) (Walters et al., 2006) had no effect on motility, frimbrae expression and growth. However, to reach a clear conclusion that AI-3 plays a role in regulating the expression of the studied factors, further studies are required.

Colonization of the small intestine by *E. coli* is characterized by its ingestion by the host and its rapid proliferation (Fairbrother et al., 2005). In this study, we found that addition of conditioned medium to *E. coli* E68 culture increased its growth rate significantly (Fig. 1). This effect began at the lag phase of culture, reaching a peak at hour four, where the highest difference among the others treatments was observed. Following this time period, the difference in relation to the control group began to reduce, and by seven hours no difference was observed among the treatments. Our findings differ from Zhu et al. (2011) which observed that after three hours of incubation, there was no significant difference in bacterial counts regardless of the initial levels of the inoculums. One might suggest that these differences could be related to the strain used, the conditioned medium concentration, temporal gene regulation and by the initial inoculum used (Freestone et al., 2007). Our data suggests that the presence of some metabolite in the conditioned medium increases the growth rate of *E. coli* E68. Thus, we propose that this effect might occur due to the presence of factor(s) (e.g., AI-3) present from the beginning of the culture, that will modulate genes responsible for bacteria growth, and the new bacteria population will produce the same factor(s), and by doing so, differentiating from the other treatments.

Interestingly, we did not observe any effect of epinephrine on bacterial growth, even when used in combination with the conditioned medium (Fig. 1), differing from other study (Sperandio et al., 2001; Bearson et al., 2010). Studies reported that incubation of ETEC with norepinephrine increased the growth rate significantly (Lyte et al., 1997). We did not observe any effect of norepinephrine (data not shown) or epinephrine regardless the concentration tested (data not shown) in the ETEC growth dynamics. A different study verified that depending on the epinephrine concentration used, it was able to inhibit *Yersinia enterocolitica* growth, and at the same concentration did not inhibit *Salmonella enterica* or *E. coli* O157:H7, suggesting that the catecholamine (norepinephrine/epinephrine) effect may be dependent on the concentration, bacterial strain and/or species studied (Freestone et al., 2007). Kendall et al. (2007) suggest that the AI-3 and epinephrine can use the same signaling pathway, and that their concentration and/or temporal presence, might play different roles in gene modulation. Thus, it might be an explanation to the observed results in the growth dynamics in our study.

It has been reported that epinephrine enhances bacterial growth by functioning as a siderophore to provide iron to the bacterial cell (Freestone et al., 2000); however, this was not observed in our work. In this study, we added iron ($FeCl_3 \cdot 6H_2O$) in the treatment that combined conditioned medium and epinephrine and we did not observe any increase in bacterial growth (data not shown). Furthermore, gene modulation by the pathway used by AI-3 and epinephrine do not necessarily overlap, suggesting that other components might interfere with this signaling pathway (Kendall et al., 2007). This observation might be relevant if it happened in the host, since the number of colonized bacteria will increase in a shorter time frame, allowing for other virulence factors (e.g., F4, LT) to be expressed in higher amounts, overcoming host defenses and leading to disease. The results of the present study provide evidence that such effect might happen in the infected ETEC host. These phenomena do not appear to be constrained to in vitro, given that litters suffering stress have been reported to increase fecal excretion of ETEC relative to control piglets (Jones et al., 2001).

The adhesion of ETEC to the intestinal epithelium is a very complex process that involves a number of structures including flagella (Bardiau et al., 2010). Flagella are complex cylindrical structures assembled from approximately 20,000 flagellin (FliC) molecules that travel down the nascent flagellar cylinder to the distal tip where they are directed by cap proteins (FliD) (Moens and Vanderleyden, 1996) into the growing flagellum. In this study we observed that the presence of conditioned medium, or its combination with epinephrine, significantly increased bacterial motility (Fig. 2). When we combined epinephrine to the conditioned medium, a significant increase in halo diameter occurred, and this effect was inhibited by the addition of β adrenergic-receptor blocker, suggesting that some signaling was through AI-3/catecholamine pathway (Fig. 2), and the qPCR findings (Fig. 4) corroborate with this observation.

Fimbriae are the primary virulence factors of ETEC, responsible for its colonization on the host. F4 fimbriae are long proteinaceous appendages composed of hundreds of identical repeating protein subunits called FaeG, as well as some minor subunits (Van den Broeck et al., 2000). The major F4 fimbrial subunit is the adhesive subunit, which allows these bacteria to adhere to specific receptors on enterocytes (Mol and Oudega, 1996), which results in colonization. Using the hemagglutination assay to detect F4 fimbriae protein expression, we observed that epinephrine alone, and conditioned medium alone do not result in an enhanced hemagglutination (Fig. 3A). However, when combined conditioned medium with epinephrine, this treatment resulted in a significant increase F4 protein expression. Similar results were observed when we used ELISA to evaluate F4 protein expression, where the treatment with conditioned medium with epinephrine showed significant protein expression (Fig. 3B). Thus, as suggested previously by others (Clarke and Sperandio, 2005) the signaling mediated by quorum sensing might have a threshold that modulates genes based on the signal and/or its intensity, and F4 gene modulation may follow this threshold.

It is possible that other molecules apart from AI-3 were responsible for the observed effects of conditioned medium. Molecules such as indole have also been described as an interkingdom signal and have added more understanding in the symbiotic interaction at the molecular level in pathogenic infections in GI tract. Since commensal *E. coli* may produce indole at concentrations that can reach 600 μM in culture (Domka et al., 2006), and indole can be detected in human feces at concentrations up to 1000 μM (Karlin et al., 1985; Zuccato et al., 1993) it is likely that pathogenic *E. coli* are exposed to this in the environment. However, it is unlikely that indole influenced the up-regulation of the genes observed in this study as Bransel et al.

(2007), reported that indole exerts an opposite effect to epinephrine and norepinephrine on EHEC motility and virulence gene expression at 37 °C. It is possible that indol had an inhibitory role that was overcome by other signaling molecules at the log phase of growth in this study.

Among the virulence factors present in ETEC, the heat-labile toxin (LT) is one of the most important, present in most isolates from post weaning diarrhea in pigs (Berberov et al., 2004; Moon and Bunn, 1993). The ETEC LT is an 84 kDa A:B5 protein structure, in which a single enzymatic A subunit is non-covalently combined with a pentamer of B subunits that bind the toxin to its receptor (de Haar and Hirst, 2004). Functions other than enterotoxicity have been ascribed to LT. Horstman et al. (2004) suggested that since LT binds to ETEC lipopolisacharide and remains combined with the bacterial surface, it can act as an adhesin binding the bacteria to the cell surface. Interestingly, Berberov et al. (2004) demonstrated that elimination of the ETEC LT genes not only reduced the severity of diarrhea but also reduced its colonization.

Following qPCR analysis of ETEC E68 cultures, we observed that all studied genes were up regulated at two hours of culture in media enriched with conditioned medium with epinephrine, and this effect was inhibited by the β adrenergic-receptor blocker, atenolol (Fig. 4A). This represents some evidence that the signaling pathway to modulate the up-regulation of these genes might be using the AI-3/catecholamines pathway. We also observed a time-dependent *fliC*, *fae*G, *fae*C and *elt*B gene mRNA expression, where an increased expression of all genes (*fliC*, *fae*G, *fae*C and *elt*B) started at 30 min and continued to increase up to two hours of culture (Fig. 4A). Interestingly, the mRNA expression of *fliC* began at low levels at 30 min, but at one hour it was expressed linearly up two hours of culture. The early expression of these genes combined with the early growth of ETEC when cultured in presence of conditioned medium and epinephrine might represent a competitive differential in the gut environment. It would be interesting to investigate if the same observation can be made *in vivo*, when host stress factor (epinephrine) and bacterial molecules (AI) are present in the same environment during the ETEC infection. AI-3 and epinephrine can use the same signaling pathway, and their threshold concentration and/or temporal presence of both modulate the expression of most important virulence factors in ETEC (F4 and LT), the presence of both in the same host environment may interfere with the out came of the disease.

5. Conclusion

Given the presence of catecholamine hormones and autoinducers in the gastrointestinal tract, findings from this study suggest that their combination within the host may increase the expression of ETEC E68 virulence factors. We observed that the presence of epinephrine and conditioned medium increases the ETEC growth rate. Additionally, their association was able to modulate the expression of the major ETEC virulence factors: F4 fimbriae and LT. The results of this study help to better understand the pathogenesis of ETEC E68 infection and suggest novel interactions that occur within the intestine to promote its virulence.

Conflict of interest

The authors declare no conflict of interest.

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