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RESEARCH ARTICLE

QseC Signaling in the Outbreak O104:H4 *Escherichia coli* Strain Combines Multiple Factors during Infection

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ABSTRACT Enteroaggregative *Escherichia coli* (EAEC) from the O104:H4 specific serotype caused a large outbreak of bloody diarrhea with some complicated cases of hemolytic-uremic syndrome (HUS) in Europe in 2011. The outbreak strain consisted in an EAEC capable to produce the Shiga toxin (Stx) subtype 2a, a characteristic from enterohemorrhagic *E. coli*. QseBC two-component system detects AI-3/Epi/NE and mediates the chemical signaling between pathogen and mammalian host. This system coordinates a cascade of virulence genes expression in important human enteropathogens. The blocking of QseC of EAEC C227-11 (Stx^+) strain by *N*-phenyl-4-{[(phenylamino) thioxomethyl]amino}-benzenesulfonamide (also known as LED209) *in vivo* demonstrated a lower efficiency of colonization. The periplasmic protein VisP, which is related to survival mechanisms in a colitis model of infection, bacterial membrane maintenance, and stress resistance, here presented high levels of expression during the initial infection within the host. Under acid stress conditions, *visP* expression levels were differentiated in an Stx-dependent way. Together, these results emphasize the important role of VisP and the histidine kinase sensor QseC in the C227-11 (Stx^+) outbreak strain for the establishment of the infectious niche process in the C57BL/6 mouse model and of LED209 as a promising antivirulence drug strategy against these enteric pathogens.

IMPORTANCE EAEC is a remarkable etiologic agent of acute and persistent diarrhea worldwide. The isolates harbor specific subsets of virulence genes and their pathogenesis needs to be better understood. Chemical signaling via histidine kinase sensor QseC has been shown as a potential target to elucidate the orchestration of the regulatory cascade of virulence factors.

KEYWORDS EAEC, *Escherichia coli*, O104:H4, QseC, Shiga toxin, VisP, chemical signaling

Some strains of *Escherichia coli* belonging to the O104:H4 serotype may be classified as members of the enteroaggregative *E. coli* (EAEC) pathovar, which was first described in the mid-1980s and has been recognized as an important cause of diarrheagenic diseases in children and adults in both developed and developing countries (1–4).

In 2011, a large outbreak of foodborne bloody diarrhea and hemolytic-uremic syndrome (HUS) began in Germany and spread throughout other countries; the outbreak was triggered by EAEC serotype O104:H4 lysogenized with Shiga toxin 2a-encoding phage, a common feature related to enterohemorrhagic *E. coli* (EHEC) or Shiga toxin *E. coli*. This outbreak emerged with more than 3,800 individuals affected, including primary and secondary cases, leading to 54 reported deaths. Moreover, the HUS cases of this outbreak occurred at a much higher rate than frequently observed for EHEC (5), i.e., approximately 22% of the cases (6, 7).

Previously, *E. coli* strains O104:H4 (Stx^+) have been reported in Europe in sporadic cases of hemorrhagic colitis and HUS (6, 8–11). However, the 2011 outbreak strain is

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genetically closer to the Stx$^-$ O104:H4 strain (55989), which was isolated from an HIV-infected patient in central Africa (12). EAEC was prevalent during a case-control study of children with diarrhea in Salvador-Bahia State, Brazil; among these subjects, an O104:H4 (Stx$^-$) strain was also isolated (13), which is genetically similar to the European isolates.

Bacterial cell-cell chemical signaling may cross over the interspecific and intraspecific differences during communication, in addition to the interkingdom signaling to their respective host cells (14). The two-component system QseBC is closely related to the expression of virulence genes in *Enterobacteriaceae*. It detects specifically AI-3 signs and adrenergic hormones epinephrine (Epi) and norepinephrine (NE) (15). QseBC and signaling via Epi/NE/AI-3 were described in EHEC (16) and in other pathogens, such as enteropathogenic *E. coli* (17), *Vibrio parahaemolyticus* (18), *Salmonella enterica* serovar Typhimurium (19–22) (among other serovars [23]), *Francisella tularensis* (24), uropathogenic *E. coli* (UPEC) (25), *Edwardsiella tarda* (26), *Haemophilus influenzae* (27), *Aeromonas hydrophila* (28, 29), *Aggregatibacter actinomycetemcomitans* (30, 31), and *Legionella pneumophila* (32).

QseC sensor kinase in EHEC is a global regulator involved in the expression of >400 genes (33), such as those encoding motility, attaching and effacing (A/E) lesions, and Shiga toxin (33–35), although QseBC's role in *in vivo* infections has never been investigated in any EAEC strain that expresses or does not express Shiga toxin.

During characterization studies of histidine kinase sensor QseC in the pathogenesis *in vivo* of *S. enterica* serovar Typhimurium, a novel periplasmic protein, VisP, was described that is associated with survival mechanisms, stress response, and the maintenance of membranes (36). VisP is important in the response to heavy metal, osmotic pressure, and acid milieu, all conditions that bacteria may encounter during gastrointestinal (GI) passage and gut colonization. In the periplasmic space of *S.* Typhimurium, VisP can bind to the sugars of the peptidoglycan layer and also to the internal membrane enzyme LpxO, which is present in *Salmonella* but absent in *E. coli*. Directly after binding, VisP blocks the specific modification LpxO-mediated 2-hydroxylation of lipid A (36). Recently, VisP was described as also involved during final O-antigen chain length regulation, together with the Wzz system from *S.* Typhimurium lipopolysaccharide (LPS) (37).

The VisP role in *E. coli* was described only in association with bacterial stress, which in turn is related to different pH conditions and stress agents such as hydrogen peroxide and cadmium chloride (38). In *S.* Typhimurium, VisP is an important player during replication within macrophages *in vitro*, systemic infection in mice, and response to the same stressors as in *E. coli* (36). Recently, VisP was described as also involved during final O-antigen chain length regulation, together with the Wzz system from *S.* Typhimurium LPS (37).

The bacterial stress response in the intestinal environment caused by bacterial pathogens leads to intestinal dysbiosis, which promotes an antagonism between the microbiota and the intruder bacteria in this system, resulting in competition for nutrients, the production of antibiotics, and modulation of the immune system (39, 40). Intestinal bacteria, in order to survive this competitive and stressful environment, must refine mechanisms that, like the pathogens, allow their permanence in the gut. Intestinal pathogens, such as EHEC, have a great ability to survive low stomach pH during GI passage, and their infecting dose is low (>100 CFU); conversely, these characteristics in EAEC were only briefly described (1, 41).

Based on EAEC diversity and its multifactorial properties described in the literature, we investigated further details in this outbreak strain and how the chemical signaling via QseC and auxiliary mechanisms are implicated in the pathogenesis of *E. coli* O104:H4. Moreover, one of the main aims of this study was to investigate the chemical signaling virulence in EAEC O104:H4 Stx$^+$ and Stx$^-$ strains by blocking QseC sensor kinase with LED209 to investigate its role during infection (42). Using *in vitro* and *in vivo* assays, we have evaluated the QseC sensor kinase importance in the outbreak O104:H4 (Stx$^+$) compared to another O104:H4 strain lacking Stx production (13). Our data show

that QseC participates in the colonization process in an animal model of colonization for EAEC and that LED209 is a potential antivirulence compound for the EAEC O104:H4 Stx$^+$ strain; these findings demonstrate the chemical signaling *in vivo* proof of principle by blocking QseC sensor kinase. During the QseC studies, compound LED209 was selected from a library of 150,000 molecules and was identified to have QseC inhibitory activity. It has been shown that the blockade of QseC by LED209 leads to the inhibition of important virulence factors in EHEC, *S.* Typhimurium, and *Francisella tularensis* (21), in addition to resulting in a higher percentage of survival among infected animals (42). Its activity in the inhibition of biofilm formation in multidrug-resistant (MDR) clinical isolates, such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, UPEC, and EAEC, was also reported.

Based on this finding, we sought to assess the chemical signaling virulence in EAEC O104:H4 Stx$^+$ and Stx$^-$ strains by blocking the QseC sensor kinase with LED209 to investigate its role during infection. This distinct approach may be considered promising in controlling the spread of pathogenic MDR microorganisms, since LED209 does not interfere with bacterial growth and consequently does not exert strong selective pressure on resistant strains. In addition, it only acts on the bacterial signal transduction pathway, does not demonstrate activity in eukaryotic cell receptors, and is not toxic in animal models. Therefore, LED209 opens perspectives for its possible clinical use in the future.

RESULTS

Chemical signaling via QseC plays an important role during O104:H4 infection. To further investigate the importance of the QseC sensor kinase in O104:H4 infection *in vivo*, mice were orally infected with 10^{10} CFU doses and monitored for 14 days. During *in vivo* assays, we evaluated mice infected with wild-type (WT) C227-11 (Stx$^+$), ::*qseC*, and BA3826 (Stx$^-$) strains in the presence or absence of LED209. Specifically, we used a C57BL/6J model, described previously for the study of EAEC intestinal colonization (43). Here, we assessed direct intestinal colonization by using CFU counts in feces to provide a numeric readout of the EAEC infection, and mouse weight loss was evaluated to investigate the effect during host regular development to correlate the infection with the disease severity (see Table S1 [https://www2.fcfar.unesp.br/#!/pos-graduacao/biociencias-e-biotecnologias-aplicadas-a-farmacia/docentes2308/corpo-docente/]). The *qseC* mutant presented lower colonization levels compared to both LED209-treated and nontreated groups; moreover, a remarkable reduction was observed during colonization at day 5 postinfection (p.i.). Clearly, treatment with LED209 led to a more evident effect during the initial days of the infection, especially between days 3 and 5 p.i., that seems to be critical for colonization and *qseC* expression. The C227-11 (Stx$^+$) strain CFU recovery was highly reduced in LED209 throughout the 14-day p.i. period (Fig. 1A). This was consistent with the observed mouse weight loss for C227-11 mice in the absence of LED209, which points out the clear effect of QseC in the Shiga toxin-producing strain (see Table S1). There was no relevant difference in CFU recovered from feces of the BA3826 (Stx$^-$)-infected mice, even though a reduction trend could be observed in the presence of LED209 (Fig. 1B). LED209 treatment reduced the number of bacteria, indicating that inhibition of QseC prevents high colonization by the C227-11 (Stx$^+$) strain.

QseC sensor kinase is critical during the initial GI passage *in vivo*. The expression levels were measured via quantitative reverse transcription-PCR (qRT-PCR) to verify *qseC* gene expression *in vitro* and during *in vivo* GI passage based on bacterial RNA extracted direct from feces collected during the mouse assay. We directly assessed here *qseC* expression during mouse infection under Shiga toxin influence in the C227-11 strain, employing Stx$^+$ and Stx$^-$ strains to evaluate QseC possible function in the process. Furthermore, the LED209-treated group displayed significant repression of the *qseC* expression at the initial days 1 and 3 p.i. of 1.6- and 1.4-fold for C227-11 (Stx$^+$) and 2.0- and 2.4-fold for BA3826 (Stx$^-$) strain in both cases, as expected since LED209 is a QseC blocker, and after 10 days p.i. the compound was degraded, and the differences

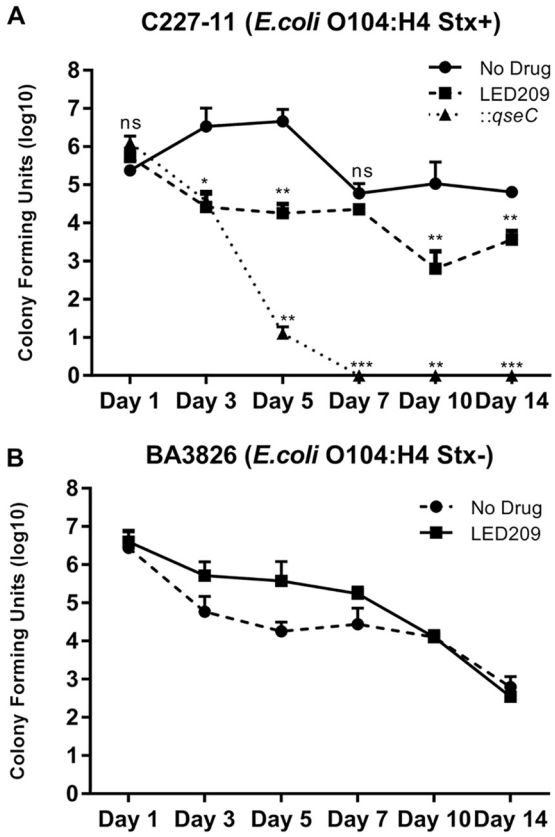

FIG 1 Mouse colonization with 10^{10} CFU of O104:H4 strains after treatment with LED209. The CFU counts for WT and ::*qseC* (C227-11) strains (A) or the BA3826 strain (B) recovered days 1 to 14 after the administration of 58.8 mg/kg of LED209 or vehicle were determined. A Student *t* test was used to determine the statistical significance between treated and nontreated groups. Error bars indicate the SD of the mean (*, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$; ns, not significant).

are surpassed or not effective anymore (Fig. 2A and B). *In vitro*, the *qseC* expression levels were very prominent in the C227-11 (Stx^+) strain, similar to EHEC 86-24 strain levels, in contrast to the BA3826 (Stx^-) strain and the other EAEC prototype strains 042 and 17-2 (Fig. 2C).

Blocking QseC shows VisP to be an important survival mechanism during mouse infection. In addition, the QseBC two-component system in the bacterial inner membrane, the VisP described in *S*. Typhimurium, participates in this process. Its role is linked to the two-component system in response to stress, general survival mechanisms, lipid A modifications, and membrane maintenance (36, 37). In EHEC, VisP seems to be important in biofilm formation in polystyrene (36). Here, we evaluated the expression levels of the *visP* gene during the course of infection, and a remarkable upregulation was observed at day 3 p.i. in both WT and ::*qseC* C227-11 (Stx^+) strains of 30.9- and 43.7-fold, respectively. However, the BA3826 (Stx^-) strain in the same time period showed only a 2.5-fold difference. Conversely, an increase in *visP* expression levels was observed in the WT and ::*qseC* C227-11 (Stx^+) strains at day 1 p.i., as well as a decrease for the BA3826 (Stx^-) strain. Together, these findings suggest that different stresses are driving VisP fine-tuning upon QseC blockade by LED209 treatment in these strains (Fig. 3A and B). Given the role of VisP in other bacterial pathogens, we further investigated similar stress conditions that O104:H4 usually overcomes during GI passage.

VisP role during O104:H4 acid stress conditions. The periplasmic protein VisP helps bacteria adapt to adverse conditions. Therefore, to verify *visP*'s importance observed *in vivo*, *visP* gene expression *in vitro* was analyzed in neutral (pH 7.2) and in

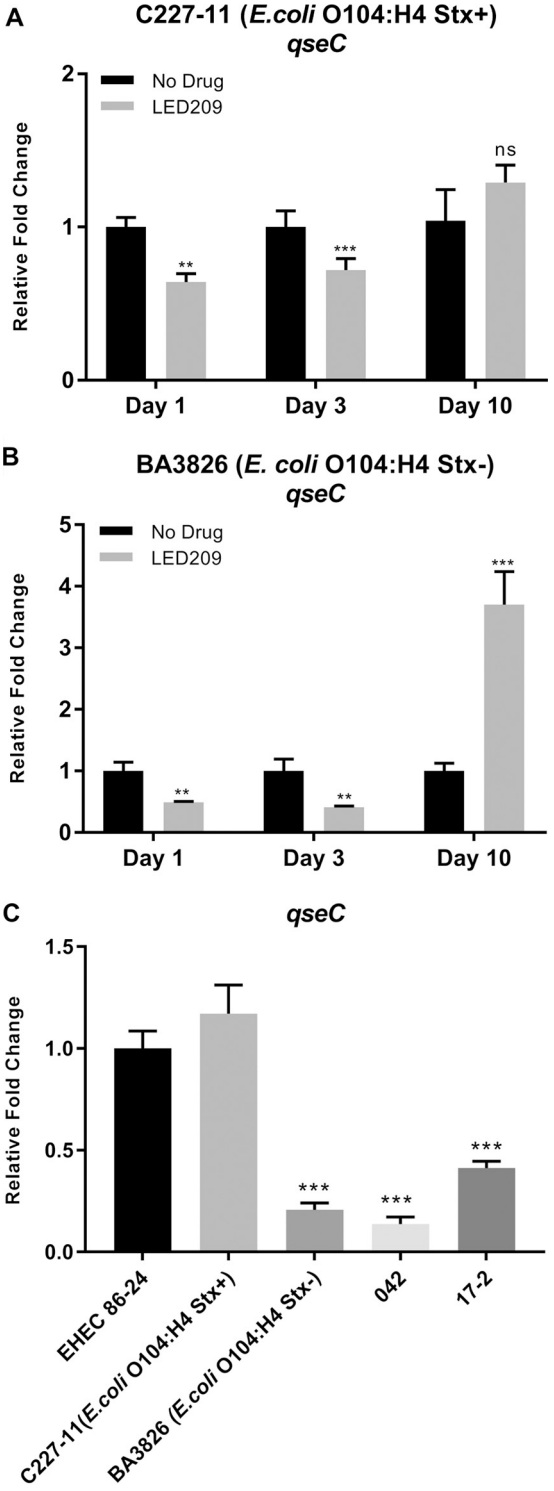

FIG 2 LED209 blocking of QseC during *in vivo* infection decreases the expression levels of *qseC* gene in O104:H4 Stx^+ and Stx^- strains. (A and B) qRT-PCR analyses of *qseC* from feces collected from days 1 to 10 p.i. of both C227-11 and BA3826 strains. (C) qRT-PCR of *qseC* with RNA extracted from cultures of EHEC 86-42, C227-11 (Stx^+), BA3826 (Stx^-), 17-2, and 042 strains grown in LB medium until reaching an OD_{600} of 1.0. A Student *t* test was used to determine the statistical significance between treated and nontreated groups, or ANOVA was used for comparisons to EHEC 86-24. Error bars indicate the SD of the mean (**, $P < 0.001$; ***, $P < 0.0001$; ns, not significant).

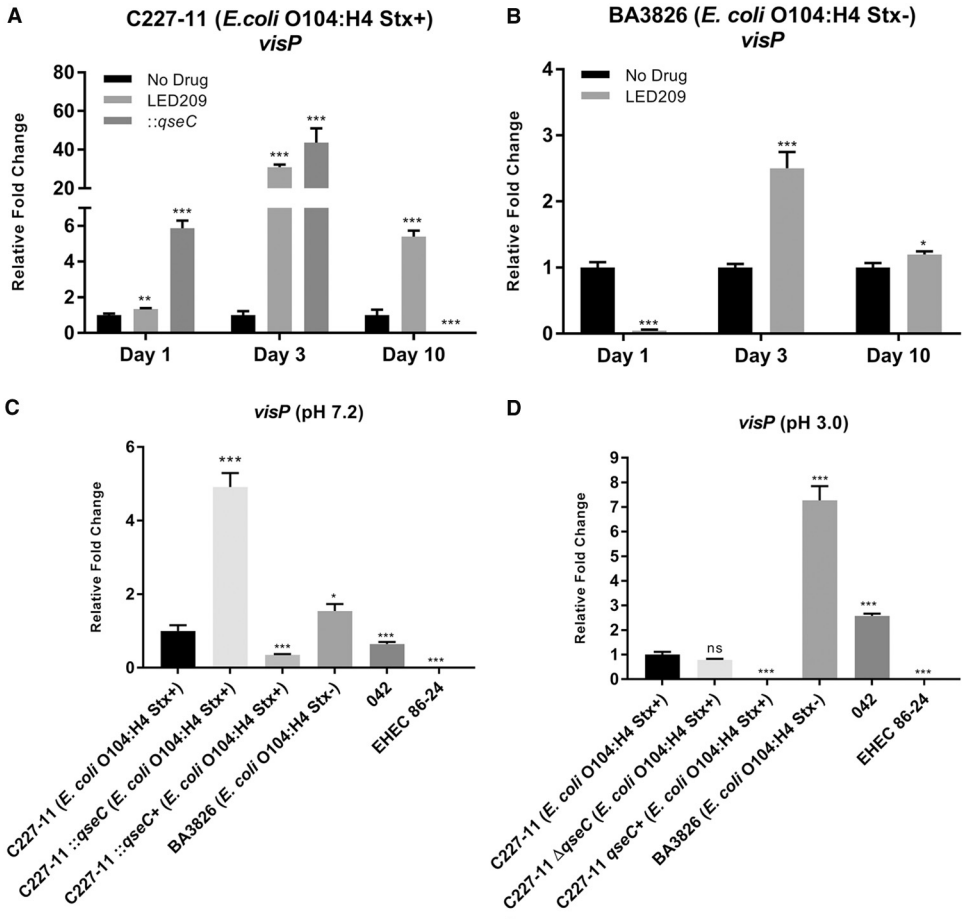

FIG 3 Stress conditions stimulate overexpression of *visP* in diarrheagenic *E. coli* strains. (A and B) qRT-PCR of *visP* from feces collected from days 1 to 10 p.i. of both C227-11 (Stx$^+$) and BA3826 (Stx$^-$) strains after treatment with LED209. (C and D) qRT-PCR of *visP* from RNA collected from cultures of WT C227-11 (Stx$^+$), ::*qseC*, *qseC*$^+$, BA3826 (Stx$^-$), 042 and EHEC 86-24 strains after 1 h of static incubation at 37°C in pH 7.2 (C) and 3.0 (D). A Student *t* test was employed to determine statistical significance between treated and nontreated groups or ANOVA comparing to WT C227-11 (Stx$^+$) strain. Error bars indicate the SD of the mean (*, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$; ns, not significant).

acidic (pH 3.0) media, and both were compared to the WT C227-11 (Stx$^+$) strain under the same conditions. In neutral pH 7.2, the ::*qseC* mutant strain showed a 4.9-fold increase, and the BA3826 (Stx$^-$) strain showed a 1.5-fold increase. On the other hand, the prototype 042 and EHEC-86-24 strains were reduced 1.5- and 81.2-fold, respectively (Fig. 3C). Upon assessing the acidic pH (pH 3.0), there was no significant difference for the ::*qseC* strain, whereas the expression of BA3826 and 042 Stx$^-$ strains increased 7.3- and 2.6-fold, and the expression of EHEC 86-24 remained low (83.7-fold) (Fig. 3D).

Taking into consideration this adaptive feature from these EAEC strains, we next evaluated the direct effect in microbiota from mice *in vivo* upon EAEC infection to better understand whether EAEC plays a role in bacterial prevalence.

***E. coli* O104:H4 shifts mouse gut microbiota upon infection.** The intestinal microbiota has been reported to play an important role in resistance to infection in the GI system, and an imbalance in its composition may enhance the ability of pathogens to survive and colonize distinct niches (44). Its composition is unique at the genus and species levels for each individual. However, at higher taxonomic levels, such as phyla, it is more conserved and is predominantly constituted by *Bacteroidetes* and *Firmicutes*, followed by *Proteobacteria* and *Actinobacteria* (45–47). Therefore, to better evaluate the impact of these EAEC strains on mice with partially depleted microbiota, we analyzed the changes in composition at the phylum level at 5 and 15 days p.i. The prevalence of

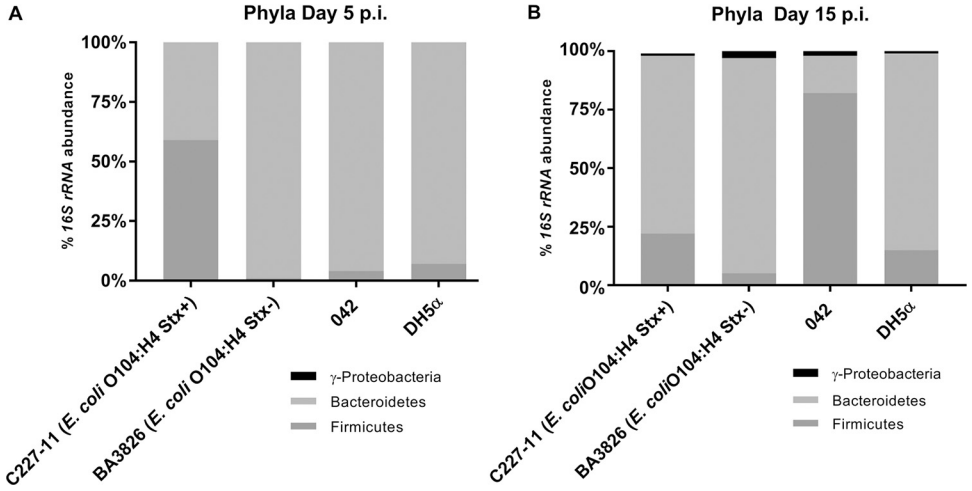

FIG 4 Gut microbiota changes in ampicillin mouse model of infection from days 5 (A) and 15 (B) p.i. with various EAEC strains. *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (more common intestinal phylogenetic groups) abundance analyses by qRT-PCR of 16S rRNA from feces were performed.

the *Firmicutes* phylum (56%) was observed on day 5 p.i. in the group infected with C227-11 (Stx$^+$) strain, while other strains showed a much higher percentage of the *Bacteroidetes* phylum (Fig. 4A). However, by 15 days p.i., relevant changes occurred, affecting the abundance of the *Firmicutes* and the reappearance of *Proteobacteria* members for all strains, especially the EAEC strains. The prevalence of *Firmicutes* was more evident (83%) for mice infected with the 042 strain compared to other strains and phyla (Fig. 4B). These results indicate the potential intestinal EAEC recolonization as one of the main hallmarks of the infection (1, 4). Other virulence factors of these multifactorial pathogens may help to elucidate their complex regulation.

Phenotypic behavior in distinct EAEC strains. The EAEC multifactorial nature was initially tested concerning its adhesion, commonly known to adhere firmly to epithelial cells and polystyrene surfaces during biofilm formation. Type I fimbriae (TIF) are an important virulence factor in UPEC (48, 49); they participate in intracellular bacterial community formation and bladder colonization in the urinary tract infection (UTI) mouse model (50). Our group has shown that TIF absence in a mutant strain led to a significant reduction in biofilm formation and aggregative adhesion (AA) pattern in EAEC 042 (51). TIF interacts with mannoside residues present in eukaryotic cell receptors (52, 53). To better evaluate its role, we blocked TIF with nonmetabolizable α-methyl-D-mannoside compound during biofilm formation essay. The results showed a significant reduction of 0.5 order of magnitude for both WT C227-11 (Stx$^+$) and BA3826 (Stx$^-$) strains after TIF blocking (Fig. 5A). The adhesion ability in HeLa cells was also evaluated, but no significant difference was demonstrated for the ::*qseC* mutant (see Fig. S1 [https://www2.fcfar.unesp.br/#!/pos-graduacao/biociencias-e-biotecnologias-aplicadas-a-farmacia/docentes2308/corpo-docente/]).

Generally, flagellum motility helps bacteria (i) to scavenge for nutrients and new colonization niches, (ii) to form biofilms, and (iii) to escape from harmful substances (54). Considered a very relevant virulence factor in several pathogenic bacteria, flagellum motility is involved in host colonization because it mediates previous contact with adhesion surface (55). In particular, type H4 flagella in UPEC are correlated with motility and epithelial cell adhesion and are prevalent in ST131 clonal type strains (56, 57). There are limited data about flagellum importance in EAEC strains.

The motility profile was identical between ::*qseC* and EHEC 86-24 strains compared to the WT C227-11 (Stx$^+$) isogenic WT strain. However, strain 042 showed a reduction of 82.3%, and strain BA3826 (Stx$^+$) showed a reduction only of 13.5% (Fig. 5B).

Are Shiga toxin and TIF involved in O104:H4 adherence? TIF analysis was performed via qRT-PCR through *fimH* gene expression, which encodes TIF pilin. *In vitro*,

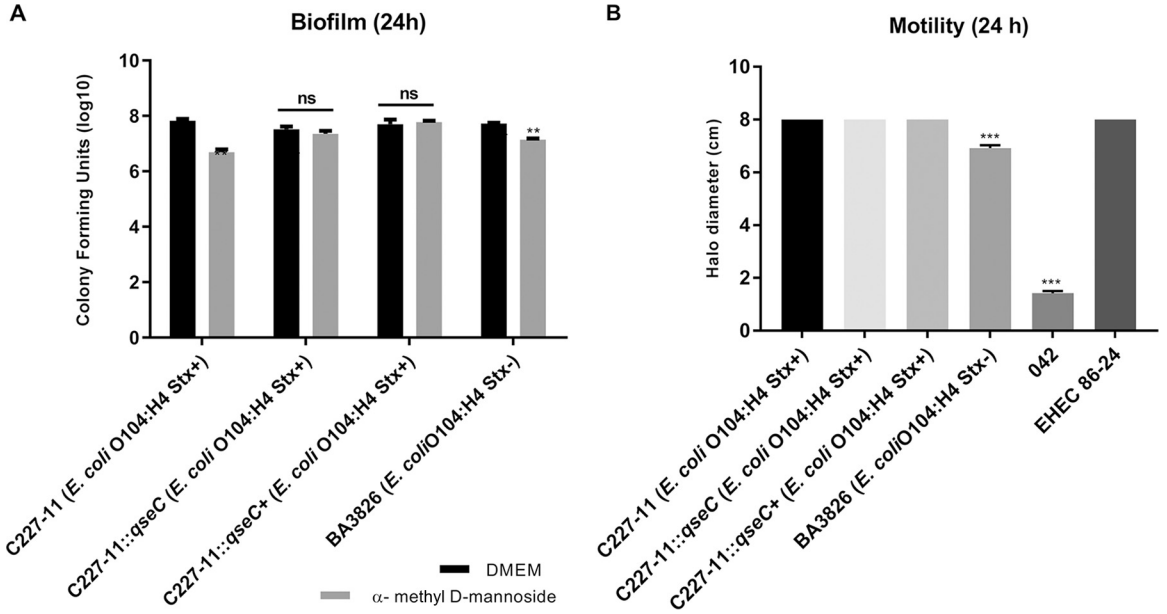

FIG 5 General phenotypic differences between EAEC strains during biofilm formation. (A) WT C227-11 (Stx$^+$), ::*qseC* C227-11 (Stx$^+$), *qseC*$^+$ C227-11 (Stx$^+$), and BA3826 (Stx$^-$) strains on 96-well polystyrene plate in the presence or absence of 1% α-methyl D-mannoside incubated for 24 h at 37°C. (B) Motility profile between *E. coli* strains compared to WT C227-11 (Stx$^+$) plated on LB agar 0.3% and incubated at 37°C. Growth halos were measured 24 h after incubation. Statistical analysis was performed using a Student *t* test between each group or ANOVA. Error bars indicate the SD of the mean (**, $P < 0.001$; ***, $P < 0.0001$; ns, not significant).

fimH was 1.7-fold higher in the WT C227-11 (Stx$^+$) strain compared to strain 042 and lower in the BA3826 (Stx$^-$) strain (Fig. 6A). *In vivo*, it was possible to demonstrate the presence of TIF at day 1 p.i. via *fimH* expression in all EAEC strains. However, the WT C227-11 (Stx$^+$) and BA3826 strains showed significant reductions of 8- and 9.7-fold, respectively, compared to the EAEC prototype strain 042 (Fig. 6B). These results indicate the presence of these fimbriae during adhesion mechanisms of different EAEC strains.

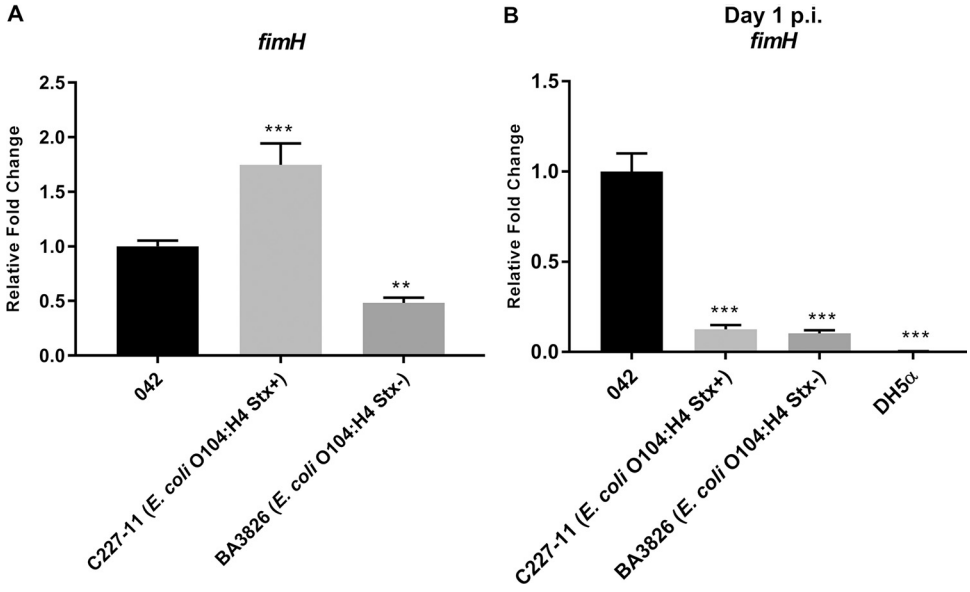

FIG 6 Role of type I fimbriae in O104:H4 Stx$^+$ and Stx$^-$ strains during *in vitro* and *in vivo* essays. (A) qRT-PCR of *fimH* of RNA extracted from 042, C227-11 (Stx$^+$), and BA3826 (Stx$^-$) cultivated strains in LB medium until reaching an OD_{600} of 1.0. (B) *fimH* expression levels determined via qRT-PCR from RNAs of EAEC and DH5α strains extracted from feces on day 1 p.i. A Student *t* test or ANOVA was used to determine the statistical significance for each replicate. Error bars indicate the SD of the mean (**, $P < 0.001$; ***, $P < 0.0001$).

Thus, TIF is notably more important for strain 042 during the early stages of infection in the C57BL/6 mouse model, although the C227-11 (Stx^+) strain exhibits lower Shiga toxin expression than EHEC 86-24 (Fig. S2) and higher expression of Stx under stress conditions (Fig. S1B), as previously shown (58). The AA pattern mediated by adherence aggregative fimbria enhances its ability to transport the toxin through epithelial cells, as previously reported (59).

DISCUSSION

The EAEC pathovar is a highly heterogeneous group of intestinal pathogenic bacteria, whereas each isolate carries an exclusive subgroup of associated virulence genes. Therefore, there is no unique virulence factor associated but rather a set of them linked to its pathogenesis (2, 60–63). The outbreak strain here studied includes a rare combination of classical EAEC features and a recently acquired Stx2a toxin.

The QseC sensor kinase detects environmental cues, such as host stress hormones, and it plays an important role in the expression of relevant virulence genes, as previously reported for important human enteropathogens (64). QseC presents a function similar to that of the G-protein-coupled receptors in the eukaryotic cell membrane, which detect stress hormones and coordinate a regulatory response cascade (33, 65).

The initial study description has shown that LED209 inhibits the autophosphorylation of QseC and directly antagonizes stress hormone epinephrine binding in this histidine kinase sensor. Moreover, *in vitro* assays with LED209 have affected the transcription of global virulence genes of EHEC, *S.* Typhimurium, and *F. tularensis* (21). Very promising results with the interference of QseC by LED209 have reduced the biofilm formation of MDR clinical isolates such as UPEC, *Klebsiella*, and *Pseudomonas aeruginosa*, as well as the EAEC O104:H4 Stx^+ strain (42). Another recent study showed the importance of LED209 as a therapeutic strategy for colitis-associated bacteria during inflammatory bowel disease caused by adherent-invasive *E. coli* (66).

LED209 hindered QseC triggering function during mouse infection by the *E. coli* O104:H4 C227-11 (Stx^+) strain; the colonization levels were higher between days 1 and 5 p.i. (Fig. 1A). Thus, LED209 during the initial colonization period, between days 1 and 3, interrupted the QseBC regulatory cascade at a critical initial point of the infection, concomitant with the higher *qseC* gene expression levels observed without LED209 blocker (Fig. 2A and B). These data corroborate with previous studies in *S.* Typhimurium- or *F. tularensis*-infected mice that have shown a significantly higher percentage of survival; in addition, these pathogens presented a lower efficiency during *in vivo* colonization when LED209 was used, especially during early infection (21, 42). Moreover, the *qseC* mutant has presented similar results to WT (C227-11) treated with LED209, a finding which concurs with the role of QseC previously reported for EHEC and *S.* Typhimurium during *in vivo* infections (19, 21, 67).

Accordingly, the increased *qseC* expression by day 10 p.i. in both O104:H4 Stx^+ and Stx^- strains (Fig. 2A and B) coincides with the beginning of colonization decline and likely to less drug availability, since LED209 was used during initial infections in these mice, as previously described (21). The QseC sensor kinase was indirectly measured *in vitro* via *qseC* gene expression levels, where both Stx^+ strains, EHEC 86-24 and C227-11, showed higher expression levels of *qseC* gene (Fig. 2C), supporting the importance of the QseC sensor kinase for these Stx^+ strains. The data presented here emphasize the relevant role of QseC kinase sensor during the course of disease caused by Shiga toxin-producing strains, such as EHEC O157:H7 infections in mouse, rabbit, and bovine models (15, 68).

VisP has an important role in response to bacterial enteric stress (36, 37), and it seems accentuated upon LED209 treatment during *in vivo* infection (Fig. 3A and B), which is consistent with a QseC-blocked role and a VisP response to stressors. Similarly, *S.* Typhimurium and the EHEC 86-24 *qseC* mutant previously presented upregulation of *visP* compared to WT levels (36). The acid pH of the stomach (<3) is one of the first barriers to be overcome during the colonization process by enteric bacteria (68, 69). In an acid milieu, such as pH 3.0 (Fig. 3D), the *visP* expression in the ::*qseC* mutant did not

demonstrate significant change, but its expression was more pronounced, especially in the EAEC Stx$^-$ strains. Therefore, this interesting role in acid stress response was observed under certain conditions for different genetic EAEC profiles in specific strains.

Recently, our group also verified that VisP is directly related to the assembly of O antigen, a highly immunogenic polysaccharide chain of LPS in *S*. Typhimurium, in addition to its important roles in macrophage survival, murine model colonization of infection, and the motility profile (37). LPS is an essential component of the cell envelope of Gram-negative bacteria and is directly related to cellular integrity, as well as in the permeability balance of the outer membrane, preventing the entry of harmful substances (70). However, the VisP role in *E. coli* LPS assembly remains unclear; the EAEC O104:H4 data here link VisP to the acid response, which may be essential during the whole process of GI tract colonization to survive the low pH of the stomach and possible undescribed LPS changes.

EHEC is a subcategory of Shiga toxin-producing *E. coli* that, in addition to Stx production, harbors the pathogenicity island LEE (locus of enterocyte effacement). LEE encodes effector proteins and a type III secretion system that are responsible for A/E histopathological lesions in the intestinal mucosa (71, 72). Although the C227-11 (Stx$^+$) strain does not harbor LEE, it presents specialized mechanisms of EAEC adherence, such as aggregative adherence fimbriae (e.g., AAF/I), which are involved in thick biofilm formation and damage to the intestinal mucosa during infection. Thus, the EAEC adherence factors, in association with Stx, result in a very successful combination of pathogenic mechanisms (6, 73, 74).

Bacterial pathogens colonize the GI tract against all host defenses in a complex and intriguing manner that raises another interesting topic regarding how enteric pathogens may change the intestinal microbiota to orchestrate colonization. EAEC has an exclusive adhesion pattern, among other diarrheagenic *E. coli*, that may present variation within the pathovar (1). Many distinct EAEC animal models such as rabbits, mice, and gnotobiotic piglets have been studied, although these animals do not represent a standard model for colonization and development of the intestinal disease caused by EAEC similar to humans (75), especially the persistent infection observed in EAEC cases (2). A recent study observed an interesting response in C57BL/6 mice upon EAEC O104:H4 (Stx$^+$) infections (43).

The intestinal microbiota plays an important role in human health maintenance, and its imbalance can lead to greater susceptibility to infection (76). All changes caused by pathogenic intruders can lead to future problems; the C57BL/6 mice here studied showed a predominance of *Bacteroidetes* phylum under all conditions evaluated. The mouse group infected with the C227-11 (Stx$^+$) strain showed a remarkable change by day 5 p.i. (Fig. 4A), characterized by an increase in the *Firmicutes* phylum, and the level of *Bacteroidetes* was reestablished in the next few days. These changes probably occurred due to the pathogens' ability to overcome barriers imposed by microbiota, which led to an exacerbated imbalance in the resident population. *Bacteroides* spp. are the main representatives of the *Bacteroidetes* phylum in the GI tract and include about 15 different species (46, 77). Members of this group, such as *B. thetaiotaomicron* and *B. vulgatus*, produce enzymes that degrade polysaccharides present in the mucus and make it available to the microbiota, which competes more efficiently for this nutrient compared to invasive pathogens such as EHEC, *Shigella*, and *Salmonella* (76). Previously, studies have shown that a significant number of *Bacteroidetes* strains encode a type VI secretion system (T6SS), and their genes are expressed in an *in vivo* infection model. Through T6SS, effector proteins may be directly injected into the cytoplasm of target bacteria, such as phospholipases, nucleases, and pore-forming proteins (77–80). Therefore, it is probable that predominance of the *Bacteroidetes* phylum has a direct or indirect connection to the resistance to the colonization of other enteropathogens that are invading the system, e.g., O104:H4 (Fig. 4).

However, other tested groups in our study did not show significant changes over the course of the study, except for the group infected with EAEC 042 strain on day 15 p.i. (Fig. 4B). An extremely high percentage of *Firmicutes*, similar to that which occurred

at day 5 p.i. with the group infected with C227-11 (Stx$^+$), was observed. Our data suggest that EAEC late colonization is indeed a feature of this *in vivo* model, similar to EAEC cases of diarrhea that can last for 14 days in humans (2). Together, these data reveal that the relative abundance of microbiota demonstrated a significant difference in the presence of C227-11 (Stx$^+$) versus other EAEC strains; this outbreak strain could damage the microbiota balance and also express Stx during the process. The inflammatory process may lead to persistent disease because of delayed repair of the intestinal mucosa and decreased absorption of nutrients, resulting in deficits in infant growth and development (2, 4, 81).

The EAEC pathology still does not completely elucidate but surely correlates to multifactorial virulence traits and vast strain differences. Therefore, aggregative adhesion is still an important standard manner to gather the strains from this pathovar. Here, we measured both cell and polystyrene adhesion between the O104:H4 strains in the presence or absence of Shiga toxin, and the toxin did not significantly affect the AA pattern (data not shown) and adherent bacterial counts.

EAEC has the important clinical features of colonizing various regions of the intestine, forming thick biofilms, and producing a large amounts of mucus (4, 41, 82, 83). Host epithelium cell adherence is an essential step during the bacterial colonization process in the gut. This process in EAEC is facilitated mainly by AAFs, followed by biofilm formation (82, 84, 85). Previous study with T84 cells has shown how AAF/I of the C227-11 O104:H4 (Stx$^+$) strain was sufficient to increase the permeability of the epithelial cell monolayer, thus contributing to an increase in Stx translocation through the epithelium (59). Besides the AAFs, EAEC encodes TIF (mannose-sensitive fimbriae), the most common adhesin found in *Enterobacteriaceae* and commonly observed in both commensal and pathogenic *E. coli* isolates. These fimbriae interact with mannose residues presented in eukaryotic cell receptors (86, 87). TIF mediates the colonization of mammalian host tissue, such as intestinal mucosa and the urinary tract. Moreover, this adhesin promotes biofilm formation on abiotic surfaces under static growth conditions. However, α-methyl-D-mannoside inhibits bacterial attachment to both biotic and abiotic surfaces because it is a direct competitive inhibitor of the mannose binding receptor by FimH adhesin of *Escherichia coli* (48, 52, 53, 88). During biofilm formation, a significant difference (by an order of magnitude of 0.5) was observed with α-methyl-D-mannoside for both strains. Together, these findings highlight the importance of TIF for biofilm formation for both C227-11 (Stx$^+$) and BA3826 (Stx$^-$) strains (Fig. 5A). Based on this, we next evaluated TIF *fimH* gene expression *in vitro* and *in vivo* (Fig. 6) to demonstrate TIF's important role, especially in the initial adhesion. However, *fimH* expression *in vivo* (Fig. 6B) is more evident for EAEC 042 than for O104:H4, consistent with previous data that emphasize the importance of TIF for this strain (51). Gene expression analysis of *stx* expressed a lower level for C227-11 (Stx$^+$) compared to EHEC 86-24 under the same test conditions, which shows an efficient Stx release by EHEC (Fig. S3). In contrast, EAEC tends to be more efficient when transporting Shiga toxin from intestinal epithelium to the bloodstream due to thick biofilm formation mediated by AAFs and TIF, factors that complicate EAEC pathogenesis. These data are consistent with a study by Zhang et al. (89), who showed that the outbreak strain of patients with hemorrhagic colitis had lost the pAA plasmid during the course of the disease in the European outbreak, which in turn caused a decrease in the ability of the EAEC O104:H4 (Stx$^+$) strain to cause HUS, since these strains become less adherent, showing the genetic plasticity of these bacteria during the course of the outbreak.

Motility has been well described in EHEC, as well as its regulation by QseC (16, 34, 90), similarly to *S.* Typhimurium mechanisms (19, 22). However, this important virulence feature has been completed neglected with regard to EAEC virulence. In the present study, a pronounced motility phenotype in both EAEC O104:H4 Stx$^+$ and Stx$^-$ strains has been observed at high levels similar to EHEC, which may be a novel interesting aspect of EAEC pathogenesis. However, the importance of flagellin and the role of flagella in EAEC pathogenesis still need to be studied more completely. Flagellum motility has been associated in many distinct pathogenic *E. coli* during the infectious

process, such as FliC flagellin H4's role in ST131 UPEC strain correlated to epithelial cell adhesion, invasion, and increased induction of cytokine interleukin-10 (56).

To summarize, QseC and VisP participate during the initial colonization process in a C57BL/6 mouse model in both O104:H4 Stx^+ and Stx^- strains. VisP seems more important for the acid response *in vitro* for the BA3826 (Stx^-) strain, during stomach-mimicking conditions, such as low pH, whereas TIF mediates adhesion mechanisms during plastic biofilm formation in EAEC strains, as briefly illustrated and explained in our current model (Fig. 7).

Therefore, our study shows that the periplasmic protein VisP is implicated in EAEC stress response and is accentuated upon LED209 treatment to block QseC during *in vivo* infection. The QseC sensor kinase in EAEC may impact mechanisms of intestinal colonization, bacterial survival, and overexpression of key chemical signaling factors. Finally, we highlight the importance of QseC in C227-11 (Stx^+) strain during colonization in an animal model and its role as a promising antivirulence drug target in similar enteric pathogens to prevent future outbreaks of Shiga toxin strain infections.

MATERIALS AND METHODS

Bacterial strains and media. All of the strains and plasmids used during this study are listed in Table 1. Strains were aerobically cultivated in Luria-Bertani (LB) broth or LB agar at 37°C. Molecular biology techniques were performed as previously described (91). All of the oligonucleotides used are listed in Table 2.

Mutant construction and complementation. The nonpolar *qseC* mutant was prepared by using suicide vector pJP5603 (92). An internal portion of the *qseC* gene of 641 pb was amplified by PCR using *qseC*-pJP5603 primers. The insert was digested with the restriction enzymes KpnI and SacI (New England Biolabs) and then inserted into the corresponding site in the suicide vector. Recombinant plasmid was transformed into *E. coli* DH5α(λ*pir*), and the colonies were selected on plates containing kanamycin (50 μg/ml). *E. coli* S17(λ*pir*) was transformed with selected plasmids or empty suicide vector as a negative control and conjugated to WT C227-11 (Stx^+) strain via filter mating on cellulose nitrate membranes. Transconjugant colonies were selected on LB agar plates containing kanamycin (50 μg/ml) and tetracycline (30 μg/ml), and integration of suicide vector pJP5603 in the *qseC* gene was confirmed by PCR analysis using the primers M13 (reverse) and *qseC*-pJP5603 (reverse). The *qseC* gene was cloned into pBAD33 (SacI/KpnI) vector and used to complement the mutant strain. The WT strain with a transformed empty vector was used in all phenotypic and genetic assays as a control for the ::*qseC* mutant. All PCRs were amplified with Platinum *Taq* DNA polymerase (Invitrogen) and 1 μM concentrations of each primer (Table 2).

Biofilm formation. Assays were performed with the O104:H4 strains WT C227-11 (Stx^+), ::*qseC* (C227-11), *qseC*$^+$ (C227-11), and BA3826 (Stx^-) to compare their abilities to form biofilm on polystyrene surface, as previously described (93). Bacteria previously grown in LB broth at 37°C were placed in 96-well plates at 1:100 in Dulbecco modified Eagle medium (DMEM) with or without α-methyl-D-mannoside and incubated for 24 h at 37°C. Finally, the wells were washed three times with sterile PBS, incubated with Tween 0.1% for 10 min, and then plated on LB agar for CFU counting.

Adherence assays. Adherence assays were performed to complement the adhesion profile (see the supplemental material [https://www2.fcfar.unesp.br/#!/pos-graduacao/biociencias-e-biotecnologias-aplicadas-a-farmacia/docentes2308/corpo-docente/]) with confluent HeLa cells as previously described (94). Cells were cultivated in DMEM (Gibco-BRL, Gaithersburg, MD) containing 10% of fetal bovine serum. Assays were performed in 24-well plates with or without α-methyl-D-mannoside (a nonmetabolizable analogue of mannose) to investigate the role of TIF in the AA pattern. Strains were grown in LB broth overnight and then used to infect HeLa cells at a multiplicity of infection of 100:1. The strains were incubated in a CO_2 incubator at 37°C for 3 or 6 h. The wells were then washed three times with sterile PBS, incubated with Triton X-100 (1%) for 10 min, and then plated on LB agar for CFU counting.

Stress response assays. For acid response assays using qRT-PCR, bacteria were statically incubated in both LB broth (pH 3.0) and PBS (pH 7.2) at 37°C for 1 h, and RNA was extracted. The acidic pH was adjusted using HCl (36).

Motility assays. The motility assays were performed using LB broth containing 0.3% agar, as described previously (14, 22). Overnight cultures grown with shaking at 37°C were inoculated at the center of each plate, followed by incubation at 37°C for 24 h, and halo diameters were measured.

qRT-PCR. RNA extraction was performed with bacteria aerobically grown in LB until reaching an optical density at 600 nm (OD_{600}) of 1.0 or fecal pellets collected from days 0 to 15 after C57BL/6J mouse infections. Then, 1 ml of TRIzol (Life Technologies) was used per 100 mg of samples (bacterial or fecal pellets). RNA was extracted by using a RiboPure bacterial isolation kit (Ambion) according to the instructions of the manufacturer. qRT-PCR was carried out with primers to detect virulence genes and predesigned RNA 16S of bacterial phyla (Table 1). QuantStudio3 (Thermo Fisher Scientific) was used for one-step qRT-PCR. Data were normalized with *rpoA* (RNA polymerase subunit A) as an endogenous control, virulence genes, and EUB (universal bacteria) for the microbiota phylum. In order to verify the relative expression of each taxon, results were normalized within day 0 (before infection). Final results were analyzed by determining the comparative critical threshold ($\Delta\Delta C_T$) method, as described previously (35), and statistical significance tests were performed using a Student *t* test.

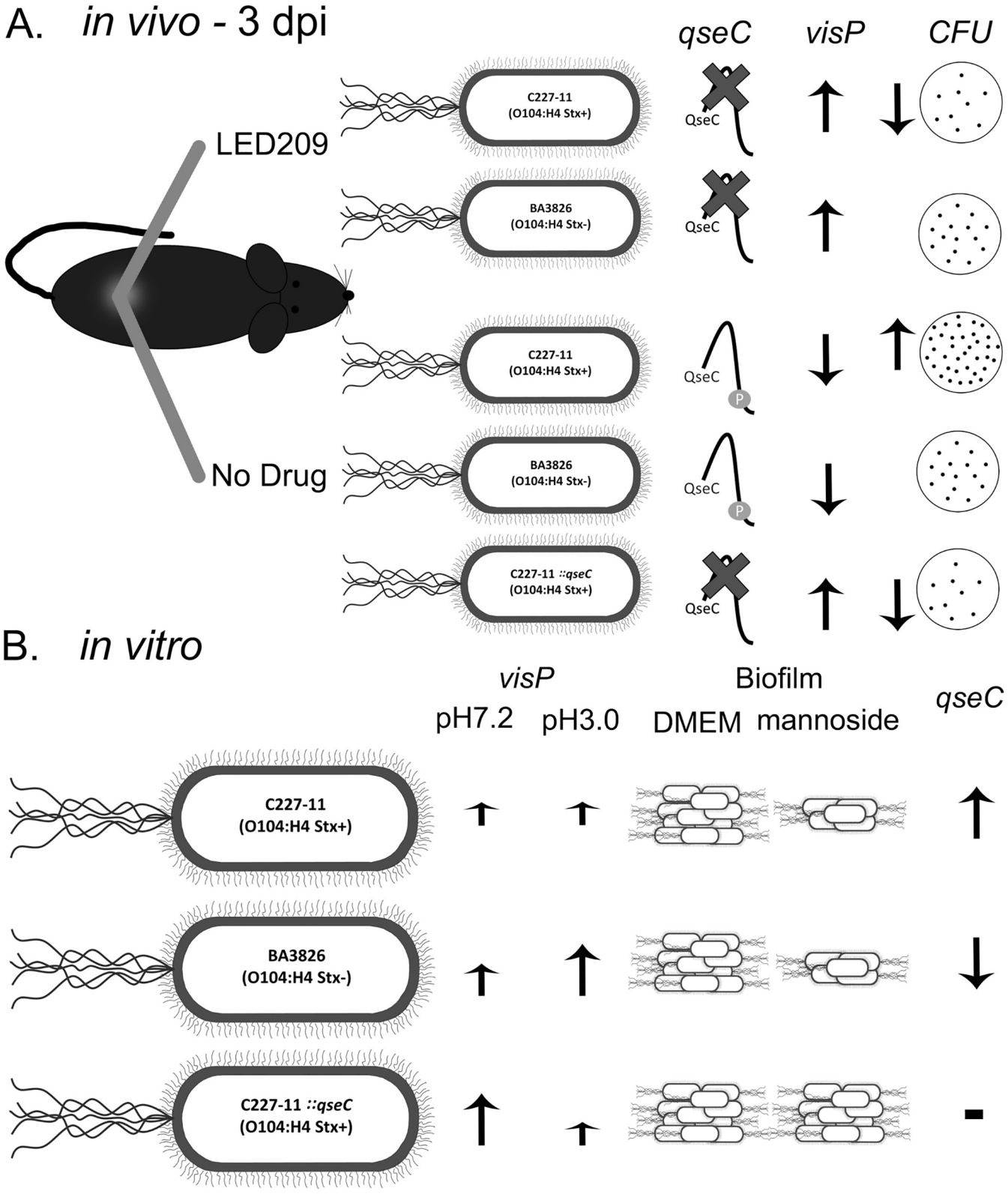

FIG 7 Proposed model for QseC and VisP role. Blocking of QseC phosphorylation by LED209 or knockout of *qseC* prevents the recognition of environmental signals and inhibits the transcription cascade of virulence genes *in vivo*. (A) Interruption of this pathway triggers other survival mechanisms, such as transcription of *visP*. (B) *In vitro*, the periplasmic protein VisP plays a role under different pH conditions between O104:H4 Stx^+ and Stx^- strains, and TIF mediates attachment on the abiotic surface.

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
86-24	EHEC O157:H7 LEE and Stx2a (isolated from outbreak of HUS in the USA)	95
WT C227-11	WT EAEC O104:H4 pAA AggR AAF/I Stx2a$^+$ Pic SigA SepA (isolated from German outbreak in 2011)	58
C227-11::*qseC*	EAEC O104:H4 *qseC* mutant	This study
C227-11::*qseC*$^+$	EAEC O104:H4 *qseC* (pBAD33) complemented strain	This study
BA3826	EAEC O104:H4 pAA, AggR, AAF/III, Pic, EAST-1 (isolated from a case-control study in Brazil)	13
042	EAEC O44:H18 pAA, AggR, AAF/II, Pet, Pic, EAST-1 (isolated from a case of diarrhea in Peru)	96
17-2	EAEC O3:H2 pAA, AggR, AAF/I, EAST-1 (isolated from a case of diarrhea in Chile)	97
DH5α	*E. coli supE44* Δ*lacU169*(ϕ80*lacZ*ΔM15) *hsdR17*	Stratagene
DH5α(λ*pir*)	DH5α transduced with λ*pir*; Nal^r	98
S17-1(λ*pir*)	Pro Res– *mod*$^+$ RP4-2 Tc::UM-Km::Tn*7* Str^r	99
TOP10	*E. coli* F$^-$ *mcrA*Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139*Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Plasmids		
pJP5603	3.1-kb R6K-based suicide vector; Km^r	92
pBAD33	Low-copy-number expression vector; Cm^r	100

[a]LEE, locus of enterocyte effacement; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Str^r, streptomycin resistance; Nal^r, nalidixic acid resistance. AAF/I, pilin subunit of aggregative adherence fimbriae I; SigA, *Shigella* IgA-like protease homologue; SepA, *Shigella* extracellular protein A; AAF/III, pilin subunit of aggregative adherence fimbriae III; EAST-1, EAEC heat-stable enterotoxin 1; AAF/II, pilin subunit of aggregative adherence fimbriae II; Pet, protease involved in intestinal colonization.

Mice. Mice were acquired from CEMIB/UNICAMP and maintained at our Animal Facility in the Biological Sciences Department at School of Pharmaceutical Sciences/UNESP. The experiment with animals was previously approved by the Animal Ethics Committee (CEUA/FCF/Car 23/2016). Animals were 3- to 5-week-old female C57BL/6J UNIB mice, weighing between 12 and 15 g. They were pretreated with 20 mg/kg of ampicillin via oral gavage, 24 h before infection, for the depletion of microbiota. Assays were divided into four experimental groups of animals with five mice per group; one group was inoculated with *E. coli* K-12 DH5a (nonpathogenic) as a negative control. The other three groups were infected, respectively, with EAEC C227-11 (Stx$^+$), EAEC BA3826 (Stx$^-$), and 042 strains. All experiments were repeated at least twice to ensure the results presented here.

Strains were cultivated for 16 to 18 h, centrifuged, and resuspended in PBS. Animals were infected with 10^{10} bacteria via oral gavage. All experiments were performed as previously described by Zangari et al. (43), except for minor modifications already mentioned. Feces were collected from mice from days 1 to 14 p.i. to recover CFU and determine gene expression by qRT-PCR. Euthanasia was performed on day 14. Mice were monitored for weight change over 14 days. Weight loss was considered for animals with a decrease of at least 5% of total body mass (43).

LED209. A total of 24 female C57BL/6J UNIB mice (3 to 5 weeks old, 12 to 15 g), received 20 mg/kg of ampicillin via oral gavage for microbiota depletion by 24 h before infection. LED209 (58.8 mg/kg) or vehicle (5% dimethyl sulfoxide, 23% polyethylene glycol 400, 70% sodium bicarbonate [pH 9], 2% Tween 80) only were administered 3 h before, at the time of, and 3 h after infection. Mice were infected with 10^{10}

TABLE 2 Primers sets used in this study

Target	Primer set sequence (5′–3′)		Source or reference
	Forward	Reverse	
qseC	CGCTGAAAGTGCAAACCGAA	CCGCGATAGAGTGAGCAGTT	This study
visP	ATGCGTTCAACGATATTGCCG	CGACCGTAGAAAGCGCAAAA	This study
fimH	CGGGGTGATGGATTCTCGTT	TCTGGGGATCTCCACCATGT	This study
rpoA	GCGCTCATCTTCTTCCGAAT	CGCGGTCGTGGTTATGTG	22
stx2a	ACCCCACCGGGCAGTT	GGTCAAAACGCGCCTGATA	21
Universal bacteria, Eub338F/Eub518R	ACTCCTACGGGAGGCAGCAGT	ATTACCGCGGCTGCTGGC	101
Firmicutes 928F-Firm/1040FirmR	TGAAACTYAAAGGAATTGACG	ACCATGCACCACCTGTC	102
Bacteroidetes 798cfbF/cfb967R	CRAACAGGATTAGATACCCT	GGTAAGGTTCCTCGCGTAT	103
Gammaproteobacteria, 1080gF/g1202R	TCGTCAGCTCGTGTYGTGA	CGTAAGGGCCATGATG	102
qseC-pJP5603	ATGGTACCCGACGGCAGAATGGTCCTTAA	GAGCTCCCTGAAGGTTATCCAGTGAGTCC	This study
qseC-PBAD33	GCAGAGCTCATGAAATTTACCCAACGTCTTAGTC	GCAGGTACCTTACATCATCACCATCACCA CCCAGCTTACCTTCGCCTC	This study
M13	GTAAAACGACGGCCAG	CAGGAAACAGCTATGAC	Invitrogen

CFU of O104:H4 C227-11 (Stx^+) and O104:H4 BA3826 (Stx^-) strains. Feces were collected from mice from days 1 to 14 p.i. to recover CFU and determine gene expression by qRT-PCR. Euthanasia was performed on day 14.

Statistical analysis. The statistical significance was evaluate by one-way analysis-of-variance (ANOVA) and Student *t* test using GraphPad Prism (v7). Experiments were repeated at least three times independently. The error bars indicate the standard deviations (SD) of the mean.

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