Expression of the locus of enterocyte effacement genes during the invasion process of the atypical enteropathogenic *Escherichia coli* 1711-4 strain of serotype O51:H40.

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Running title: LEE gene expression during aEPEC invasion and persistence

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Abstract

Atypical enteropathogenic *Escherichia coli* (aEPEC) is a significant cause of diarrhea in developing countries. Some aEPEC strains, including the Brazilian representative strain of serotype O51:H40 called aEPEC 1711-4, can use flagella to attach to, invade, and persist in T84 and Caco-2 intestinal cells. They can even translocate from the gut to extraintestinal sites in a rat model. Although various aspects of the virulence of this strain were studied and the requirement of the T3SS for the efficiency of the invasion process was demonstrated, the expression of the LEE genes during the invasion and intracellular persistence remains unclear. To address this, the expression of flagella and the different LEE operons was evaluated during kinetic experiments of the interaction of aEPEC 1711-4 with enterocytes *in vitro*. The genome of the strain was also sequenced. The results showed that flagella expression remained unchanged, but the expression of *eae* and *escJ* increased during the early interaction and invasion of aEPEC 1711-4 into Caco-2 cells, and there was no change 24 hours post-infection during the persistence period. The number of pedestal-like structures formed on HeLa cells also increased during the 24-hour analysis. No known gene related to the invasion process was identified in the genome of aEPEC 1711-4, which was shown to belong to the global EPEC lineage 10. These findings suggest that LEE components and the intimate adherence promoted by intimin are necessary for the invasion and persistence of aEPEC 1711-4, but the detailed mechanism needs further study.

Importance

Atypical enteropathogenic *Escherichia coli* (aEPEC) is a major cause of diarrhea, especially in developing countries, like Brazil. However, due to the genome heterogeneity of each clonal group, it is difficult to comprehend the pathogenicity of this strain fully. Among the aEPEC strains, 1711-4 can invade eukaryotic cells *in vitro*, cross the gut barrier, and reach extraintestinal sites in animal models. By studying how different known aEPEC virulence factors are expressed during the invasion process, we can gain insight into the commonality of this phenotype among other aEPEC strains. This will help us develop preventive measures to control infections caused by invasive strains. No known virulence-encoding genes linked to the invasion process were found. Nevertheless, additional studies are still necessary to evaluate the role of other factors in this phenotype.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) is still an important pathogen related to diarrheal diseases in developing countries. In Brazil, it is the most frequent *E. coli* pathotype isolated from diarrhea (1). The EPEC pathotype is subdivided into two subgroups, typical and atypical, based on the production of the adhesion factor named bundle forming pilus (BFP), present on typical EPEC (tEPEC) and absent on atypical EPEC (aEPEC) (2). Due to the absence of BFP, aEPEC requires a prolonged time of interaction with enterocytes *in vitro* to adhere to and promote attaching and effacing (A/E) lesion (2) that is mediated by proteins encoded by genes present in the Locus of Enterocyte Effacement (LEE) pathogenicity island. The A/E lesion is characterized by the intimate adherence between bacteria and host cells, which promotes microvilli reshuffling into pedestal-like structures. Such cell surface modification is promoted by the injection of diverse proteins encoded in the LEE region

through the type three secretion system (T3SS), which result in the mobilization of actin and other cytoskeletal proteins to the adherence site (2–4).

The LEE comprises five polycistronic operons (LEE1, LEE2, LEE3, LEE4, and LEE5), two bicistronic operons (*espG-rorf1* and *grlA-grlR*), and four independent genes (*etgA*, *cesF*, *map*, and *escD*), and together with diverse LEE-independent genes, are directly involved in the A/E lesion formation and diarrhea caused by the EPEC pathotype (5, 6).

The aEPEC subgroup is genetically heterogeneous (4, 7–9) and different strains may carry additional virulence factors that might contribute to the initial stages of enterocyte colonization and diarrhea. Strains belonging to the O51:H40 serotype comprise one of the most frequent among aEPEC isolated from diarrhea in Brazil (1, 10–13). One such strain, aEPEC 1711-4 was used in various previous studies focused on enlarging our knowledge of the virulence of the aEPEC strains. In these studies, we showed that this strain could use flagella to attach to enterocytes during the initial colonization (14), and was able to invade and persist inside T84 and Caco-2 cells (15), induce IL-8 (14, 15) and translocate from the gut to different extraintestinal sites causing systemic infection in a rat model (16). Sampaio et al. (17) showed that the 1711-4 isogenic strain lacking the T3SS was not able to invade Caco-2 cells properly, to translocate from the gut to extraintestinal sites or promote A/E lesion in a rabbit ileal loop model, demonstrating the relevance of this system in all these phenotypes. Although various aspects of the virulence of this strain were studied and the requirement of the T3SS for the efficiency of the invasion process was demonstrated, the impact of the LEE genes expression during the invasion and intracellular persistence remains unclear; additionally, full details regarding the 1711-4 strain's genome are unknown. Therefore, in this study, we evaluated the expression of the LEE regions and flagella during

98 the invasion and persistence process of aEPEC 1711-4 into Caco-2 and HeLa cells. We also
99 provided the whole sequence and analyses of the aEPEC 1711-4 genome.

100

101 **Methods**

102 **Bacterial strain**

103 The aEPEC 1711-4 strain (serotype O51:H40) was isolated from a child with diarrhea during
104 an epidemiological study on diarrhea, which was conducted in 1989 at the Universidade
105 Federal de São Paulo (UNIFESP), Brazil (13).

106 One fluorescent variant of aEPEC 1711-4 was obtained by transforming the wild-type strain
107 with the recombinant plasmid pDP151 (Invitrogen), which encodes the fluorescent protein
108 mCherry and confers resistance to ampicillin.

109

110 **Cell culture**

111 Caco-2 cells ATCC® HTB-37™ were used to evaluate differential expression of the LEE
112 genes during the invasion process and intracellular persistence. The cells were cultivated in
113 24-well plates using Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA)
114 supplemented with 10% Fetal bovine serum (Gibco, USA), 1% antibiotic mixture (penicillin-
115 10,000 U/ml and streptomycin-10 mg/ml, ThermoFisher, USA), and 1% non-essential amino
116 acids mixture (Life Technologies, USA), in an atmosphere of 5% CO_2 at 37°C for up to 10
117 days, to enable cell polarization and differentiation.

Before the assays, the monolayer was washed three times with phosphate-buffered saline (PBS), and fresh DMEM supplemented with 2% FBS was added.

HeLa cells stably expressing Lifeact::GFP (18, 19) were used to evaluate the formation of pedestal-like structures and invasion in HeLa cells. The cells were grown in DMEM with 10% FBS, 1× PSN, 50 µg/ml of hygromycin B (ThermoFisher, USA), and kept in a 5% CO_2 atmosphere at 37 °C. For the fluorescence microscopy assay, HeLa(actin-GFP) cells (5×10^5 cells/per dish) were seeded onto 35 mm cell culture dishes with glass bottom (Corning, USA) 48-h before the assay.

Evaluation of the bacterial invasion and intracellular persistence.

The invasion assay was performed as described by Pacheco *et al*. (20) with modifications. Briefly, overnight cultures grown in Lysogeny broth (LB) were adjusted to ~ 0.5 OD_{600}, inoculated in a ratio of 1:50 in two 24-well plates containing polarized and differentiated Caco-2 cells, and incubated at 37 °C for 1.5 h, 3 h, and 6 h. After the incubation period, one plate was washed three times with PBS, 1 ml of DMEM containing 2% FBS. Then, 100 µg/ml of gentamicin was added to each well and plates were incubated for 1 hour to kill extracellular bacteria. The other plate was washed and kept untreated. After incubation, cells in both plates were lysed with 1% Triton X-100, serially diluted, plated onto MacConkey agar plates, and incubated at 37°C for 18 h to determine the numbers of total bacteria (TB) and intracellular bacteria (IB). The invasion index was calculated as (IB × 100 / TB). The assays were performed in biological and technical triplicates, and results were presented as mean ±standard deviation.

The evaluation of bacterial persistence was performed as the invasion assay. However, after 6 h of incubation, the cells were washed three times with PBS, 1 ml of DMEM supplemented with 2% FBS and 100 µg/ml of gentamicin was added to each well, and the preparations were incubated at 37°C for 18 h, totalizing a 24 h assay. After incubation, the monolayers were washed with PBS, lysed with Triton X-100, serially diluted, and plated onto MacConkey.

Fluorescence actin staining (FAS) assay and F-actin pedestal quantification

The assay was performed as previously published. Briefly, aEPEC 1711-4 expressing mCherry was grown statically in LB for 18 h at 37°C. HeLa (Actin-GFP) cells in DMEM supplemented with 2% FBS and 100 µg/ml of ampicillin were infected with 1.5×10^7 bacteria prepared from an overnight culture. Pedestal formation was evaluated after 1.5 h, 3 h, and 6 h of incubation at 37 °C in an atmosphere of 5% CO_2. The assay was washed with PBS and fixed with 3.7% formaldehyde. Then, the coverslips were washed with PBS, incubated with 8 µM of FITC-phalloidin (Invitrogen), washed with PBS and Saline-Sodium Citrate buffer (SSC) [2×], treated with 100 µg/ml RNAseA (Sigma-Aldrich), washed with SSC 2×, incubated with 1.7 µM of propidium iodide, and rewashed with SSC 2×. The cells were visualized with a Zeiss confocal microscope with a 63x1.40 N.A. immersion oil objective. Pedestals were quantified by randomly imaging different fields while recording the number of cells showing F-actin accumulation foci. Results were presented as means of percentage (%) of infected cells with F-actin accumulation or number of pedestals per cell ±standard deviation. The assay was assessed in replicates and at least two independent experiments.

qRT-PCR

The expression of the *ler, escJ, escV, escN, eae,* and *espA* genes*,* representing the different operons in the LEE region, and *fliC*, encoding the bacterial flagellin*,* was evaluated during the infection and persistence assays by qRT-PCR. For that, invasion and persistence were performed using Caco-2 cells seeded into 6-well plates. The monolayer was lysed at each specific time for invasion (90 min, 3 h, and 6 h) and persistence (6 h and 24 h), and total RNA was extracted using the RNeasy Plus Mini kit (Ambion, Lithuania). The RNA was quantified using a Biophotometer (Eppendorf, Germany), DNA was removed using DNAse (Invitrogen, USA), and the reverse transcription was performed using Superscript FirstStrand Synthesis for RT-PCR (Invitrogen, CA, USA). cDNA was amplified using the Master Mix kit (Thermofisher, USA) with specific primers designed (Table 1). *rpoA* was used as a gene expression control.

Whole genome sequencing and genomic analyses

The whole genome sequence of 1711-4 was performed using the PacBio RS sequencing System (Pacific Bio). The reads were assembled *de novo* using Canu assembler v 1.7.1 (25) and polished using Racon v1.4.13 (26). The assembled genome was used to perform diverse analyses at the Center for Genomic Epidemiology (CGE: http://genomicepidemiology.org/) using services for the identification of virulence genes (VirulenceFinder version 2.0)(27, 28), serotype (SeroTypeFinder version 2.0)(29), antibiotic resistance genes (ResFinder version 4.1)(30, 31), plasmids (PlasmidFinder version 2.0)(32), and sequence type determination (MLST version 2.0)(33), following the Warwick scheme. The virulence factors were also searched using the virulence factors database (VFDB)(34).

185 The Similar genome finder service with a threshold of 0.001 and distance of 0.01 was used
186 to identify the published genomes like 1711-4 to identify to which Clonal complex the strain
187 belonged. The MLST of the 50 *E. coli* strains similar to 1711-4 were evaluated in the CGE
188 as described above. The clonal complex of the strains was searched using EnteroBase
189 (https://enterobase.warwick.ac.uk/) (35). To understand their relationship, a phylogenetic
190 tree was built using the Bacterial Genome Tree Service (36) with all the strains. To build the
191 tree, prototype *E. coli* O127:H6 str. E2348/69 (ST15-B2), O157:H7 str. Sakai (ST11-E),
192 O104:H4 str.2011C-3493 (ST678-B1), and H10407 (ST10-A) were used as outgroups.

193 Another phylogenetic tree was built to identify the clonal relationship of 1711-4 with EPEC
194 and EHEC clonal lineages previously published (9, 37) at the BV-BCR. The trees´ final
195 layouts were built using iTOL v 6.8 (38).

196

197 **Statistical analyses**

198 The results were compared and evaluated using the non-parametrical t-student test. The
199 numbers of FAS-positive signals and pedestals were compared using One-way ANOVA
200 followed by the post hoc Turkey HSD test. P values ≤ 0.05 were considered statistically
201 significant. The statistical analyses were performed using Prism GraphPad ver. 8.4.2.

202

203 **Results**

204 **Adhesion and invasion of aEPEC 1711-4 with epithelial cells occur at different time**
205 **points.**

206 A kinetic interaction was performed to understand the 1711-4 adhesion and invasion
207 behaviors in the colonization of Caco-2 cells. The number of bacteria interacting with Caco-
208 2 cells increased from 1.5 h to 3 h ($P \leq 0.0001$) but did not change between 3 h and 6 h
209 (Figure 1A).

210 Although the total interaction did not change, the quantification of the invasion efficiency in
211 the same period showed that the aEPEC 1711-4 invasion process occurred only after 1.5 h
212 and increased after 3 h of interaction (Figure 1B).

213

214 **Expression of *eae* and *escJ* increases during the invasion process in Caco-2 cells but**
215 **does not change during the intracellular persistence.**

216 As the transcription levels of the LEE genes during the initial interaction of aEPEC 1711-4
217 with Caco-2 cells is unknown, the *ler, escJ, escV, escN, eae,* and *espA* transcription levels
218 were evaluated to verify the impact of their operons in the process. Additionally, as it is
219 known that flagella participate in the initial interaction of 1711-4 with Caco-2 cells (14), the
220 transcription of *fliC*, encoding the flagellin, was also evaluated.

221 The transcription of the *escJ* and *eae* genes from aEPEC 1711-4 gradually increased during
222 the first 6 hours of interaction with Caco-2 cells, while the transcription of the other genes
223 evaluated did not change along the period (Figure 1C).

224 During the bacterial intracellular persistence, although *ler*, *escJ*, and *escN* displayed higher
225 levels of transcription in 24 h than in 6 h, there was no notable change in expression of the
226 genes, comparing 6 h and 24 h (Figure 1E). Similarly, the number of bacteria inside the
227 Caco-2 cells barely changed from 6 h to 24 h (Figure 1D).

Pedestal formation and bacterial invasiveness increase along the interaction with HeLa cells

The number of pedestals per cell increased gradually from 3 h to 6 h (Figure 2, Figure 3A), being significantly higher at 6 h (Figure 3A). In the same way, the invasion of HeLa cells reached 12% at 6 h but internalized bacteria were scarce 3 h post-infection (Figure 3B).

aEPEC 1711-1 belongs to the EPEC global lineage 10, ST10 complex, and phylogroup A.

The whole genome of aEPEC 1711-4 comprised 4,722,189 bp and belonged to phylogroup A. Also, the serotype O51:H40 was confirmed *in silico* but the MLST ST was not identified. To detect to which clonal complex the strain belonged, Mash/MinHash was used to screen the genomes similar to aEPEC 1711-4 and deposited at the NCBI. The strains identified belonged mainly to the ST10 (43/51), two belonged to the clonal complex of sequence type 10 (ST10 cplx) (ST34 and ST752), and six strains belonged to underrepresented STs (ST5332, ST5339, and ST5353) not assigned to any clonal complex but that clustered together with the strain from ST10 cplx. Most strains identified in the cluster belonged to the aEPEC pathotype and were isolated worldwide (Figure 4 and Supp Table 1).

Additionally, the evaluation of the EPEC/EHEC global clonal lineages showed that aEPEC 1711-4 belongs to the EPEC10 clonal group (Figure 5).

Regarding virulence factors, aEPEC 1711-4 carried a complete LEE region and the intimin subtype theta. The non-LEE effectors NleB1, NleB2, NleE1, and NleH1 and the adhesins

ECP, ELF, FdeC, YDF, and HCP were also identified in the genome. No known invasin, plasmid, or genes related to extraintestinal pathogenicity or antimicrobial resistance were identified in the aEPEC 1711-4 genome.

Discussion

The aEPEC pathotype comprises genetically heterogeneous bacteria that harbor diverse accessory genes that contribute to their virulence (7–9). In contrast with the tEPEC pathotype, the mechanisms related to aEPEC pathogenicity are not well established due to their considerable diversity. Furthermore, aEPEC is still one of the major causes of diarrhea in developing countries like Brazil, being the most prevalent diarrheagenic pathotype isolated from diarrheal diseases (1).

In the present work, we enlarged the knowledge regarding the 1711-4 strain, one representative Brazilian aEPEC strain belonging to the O51:H40 serotype. As the expression of flagella and the LEE PAI genes in the invasion and intracellular bacterial persistence was unknown, we evaluated the expression of five LEE genes, representing the different LEE regions, and flagella during the early invasion and persistence processes in Caco-2 cells.

During the interaction of aEPEC 1711-4 with Caco-2 cells, the number of associated bacteria reached its maximum at 3 h, while invasiveness was identified only after 3 h. During this process, the *escJ* and *eae* genes continuously increased their expression rate, reaching their maximum 6 h post-infection. This increase suggested that the proteins encoded by these genes might be involved in the invasion process. The *eae* gene codes for the adhesin intimin, responsible for the bacterial intimate adherence to host cells and represents the LEE 5 region.

In contrast, *escJ* represents the LEE 2 region and codes for one of the internal membrane structural proteins of the type 3 secretion system (T3SS), which is responsible for the injection of all effectors involved in A/E lesion formation, including the secretion of Tir, the intimin receptor that is also encoded in the LEE 5 region (2, 21, 39).

The T3SS is an essential virulence factor related to the pathogenicity of diverse bacterial genera like *Shigella*, *Salmonella,* and *Yersinia* (39–42). Studies showed that it is expressed constitutively in some pathogens like *Shigella*, although the T3SS proteins are only produced when the bacteria interact with the host cell membrane (43–45). Additionally, the T3SS expression is repressed once *Shigella* is released in the cytosol (43).

During the interaction with HEp-2 cells, it was observed that the expression of LEE operons in the tEPEC prototype strain E2348/69 was different from that of aEPEC 1711-4 infection of Caco-2 cells (46). In tEPEC, the *ler* gene was repressed at 3 hours post-infection, while the expression of *tir* and *eae* increased after 3 hours, and *espA* after 5 hours. However, there was no notable change in the expression of *escV*. In contrast, during aEPEC 1711-4 infection of Caco-2 cells, no significant change in the expression of the *ler*, *escN*, and *espA* genes was found during the studied period of up to 24 hours.

Some results were similar to those reported with tEPEC (46), such as the absence of significant change in *escV* expression and an increase in *eae* expression at 3 hours post-infection. However, in tEPEC, no increase in *eae* expression was identified after 5 hours, while in aEPEC 1711-4, *eae* expression kept increasing up to 6 hours post-infection and maintained the expression rate until 24 hours. It is interesting to note that in *E. albertii,*

294 another invasive pathogen that harbors the PAI LEE, the expression of the LEE regions also
295 differed from that identified in the present study. In the *E. albertii* strain 1551-2, there was
296 no difference in the expression of any of the evaluated genes (*ler, escJ, escV, escN, eae,* and
297 *espA*) at 1.5 h, 3 h, and 6 h post-infection (22), while all genes but *espA* showed an increase
298 on gene expression at 24 h post-infection (22). This comparative and complex scenario
299 perhaps indicates that the expression of the LEE genes can be differentially regulated in
300 different isolates, and perhaps this fact can lead to differences in their virulence potential.

301 In aEPEC 1711-4, the differences in the gene expression identified may be linked to the
302 invasion and/or persistence processes. In a previous study, it was demonstrated that the
303 aEPEC 1711-4 isogenic strain lacking T3SS is unable to invade eukaryotic cells. Now, we
304 have found that in the wild type 1711-4 strain, the *escJ* and *eae* genes are upregulated during
305 the invasion process, and this expression remains high during the persistence stage. This
306 supports the notion that LEE components play a role in the invasion process. Further analysis
307 is needed to determine the role of individual LEE components in the invasion process of
308 aEPEC 1711-4. Additionally, we confirmed that different from other LEE harboring invasive
309 strains, aEPEC 1711-4 cannot multiply intracellularly but remained viable up to 48 h post-
310 infection without an increase in the intracellular population (14, 20).

311 Our group previously showed that the flagella of aEPEC 1711-4 contribute to the early steps
312 of bacterial adherence (14). For EHEC strains, it has been demonstrated that the *fliC* gene is
313 repressed during the infection process (47). In the present study, we showed that *fliC*
314 expression is continuous up to 24 h after infection, indicating that *fliC* continues to be
315 expressed even after bacterial adherence to the cell surface for unknown reasons.

316 It was recently discovered that the 1711-4 strain lacks plasmids and belongs to the ST10
317 complex and EPEC 10 global lineage. This lineage is associated with EPEC strains that cause
318 diarrhea and extraintestinal infections. The global EPEC 10 lineage, to which 1711-4
319 belongs, represents about 15% of all aEPEC strains isolated worldwide and is the most
320 significant global lineage in phylogroup A. The genome of the 1711-4 strain was evaluated,
321 and it was found to contain non-LEE effectors (NleB1, NleB2, NleE1, and NleH1) as well
322 as five adhesins (ECP, ELF, FdeC, YDF, and HCP). However, no invasin or genes related to
323 extraintestinal pathogenicity were identified that could explain its ability to invade cells and
324 move from the gut to extraintestinal sites. The role of these factors in bacterial adherence,
325 invasion, and host cell subversion remains to be determined.

326

327 **Data availability**

328 This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank
329 under the accession JAVKVK000000000. The version described in this paper
330 is version JAVKVK010000000

331

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Author contribution

Conceptualization: TATG and RTH; Data Curation: TATG; Formal Analysis: FTR, RTH and ACMS; Funding acquisition: TATG; Investigation: FTR, ACMS; Project Administration: FTR; Resources: VS and TATG; Supervision: RTH, VS, and TATG; Validation: FTR, RTH and ACMS; Visualization: FTR and ACMS; Writing – original draft: ACMS; Writing – review and editing: ACMS, RTH, TATG. All the authors read the manuscript's final version and agreed with the manuscript submission.

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513

514

515 **Table 1**: Primers used in RT-PCR

Primers	Sequence	Region	Reference
***ler* (F)**	CGACCAGGTCTGCCCTTCT	LEE 1	(21)
***ler* (R)**	GGGCGGAACTCATCGAA	LEE 1	(21)
***escJ* (F)**	GGCGATGCCACTAACTGACT	LEE 2	(22)
***escJ* (R)**	GCAAGCACTGTTGCTATCCA	LEE 2	(22)
***escV* (F)**	GGCTCTCTTCTTCTTTATGGCTG	LEE 3	(23)
***escV* (R)**	CCTTTTACAAACTTCATCGCC	LEE 3	(23)
***escN* (F)**	GATTTCCCCCGAGTGTTTTT	LEE 3	(22)
***escN* (R)**	CTGCAAGTTCTCGGGTAAGC	LEE 3	(22)
***eae* (F)**	TCGATATCCGCTTTAATGGC	LEE 5	Present study
***eae* (R)**	CCCGTACCATGACGGTAATC	LEE 5	Present study
***espA* (F)**	TAAGGAGTCAACCACTGCCC	LEE 4	Present study
***espA* (R)**	AAATCACCAGCGCCTAATTG	LEE 4	Present study
***fliC* (F)**	CATTCAGCGCACCTTCAGTA	Flagellin	(22)
***fliC* (R)**	TCTCTTCTGGCCTGCGTATT	Flagellin	(22)
***rpoA* (F)**	GCGCTCATCTTCTTCCGAAT	control	(24)
***rpoA* (R)**	CGCGGTCGTGGTTATGTG	control	(24)

516

517

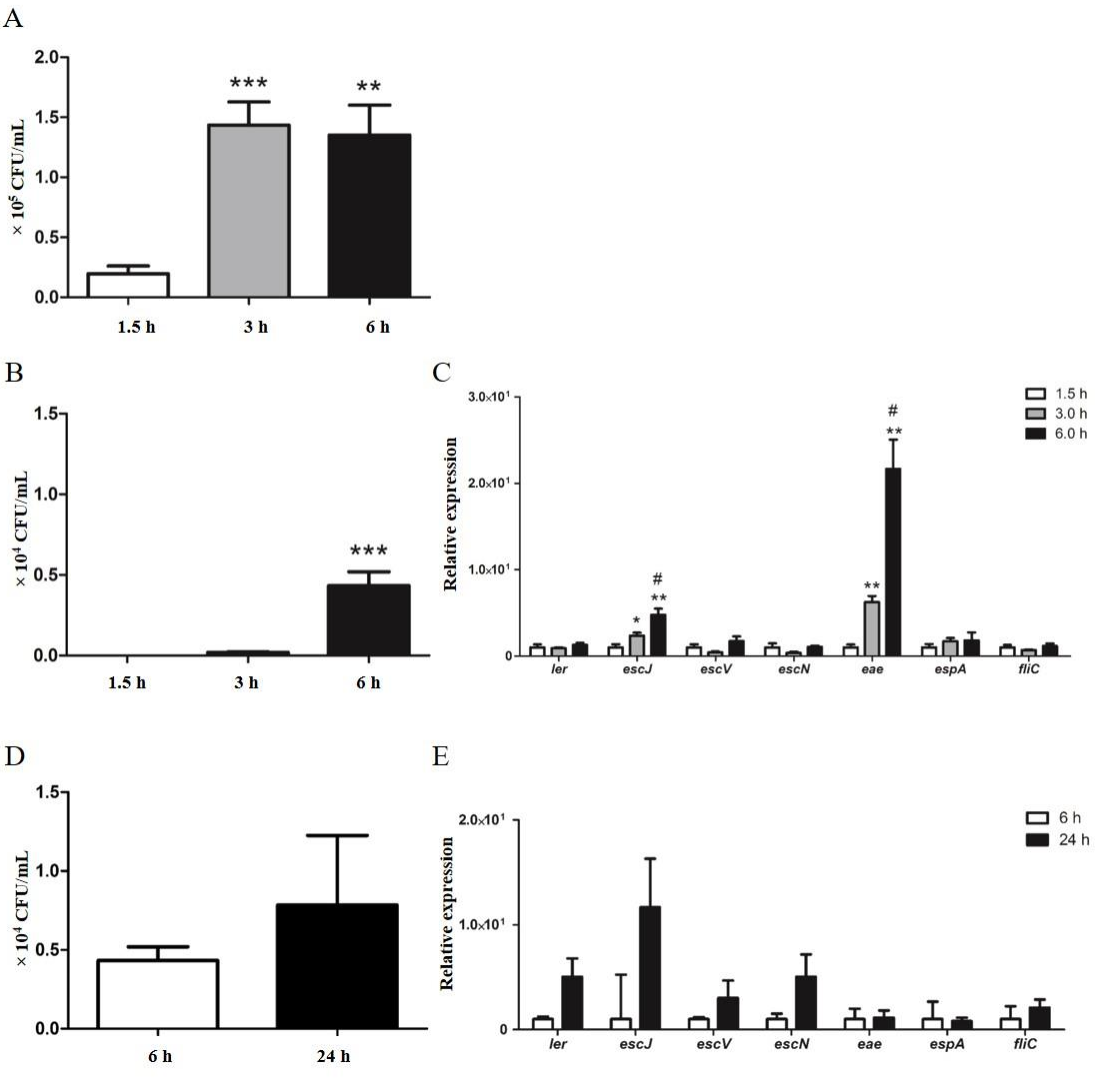
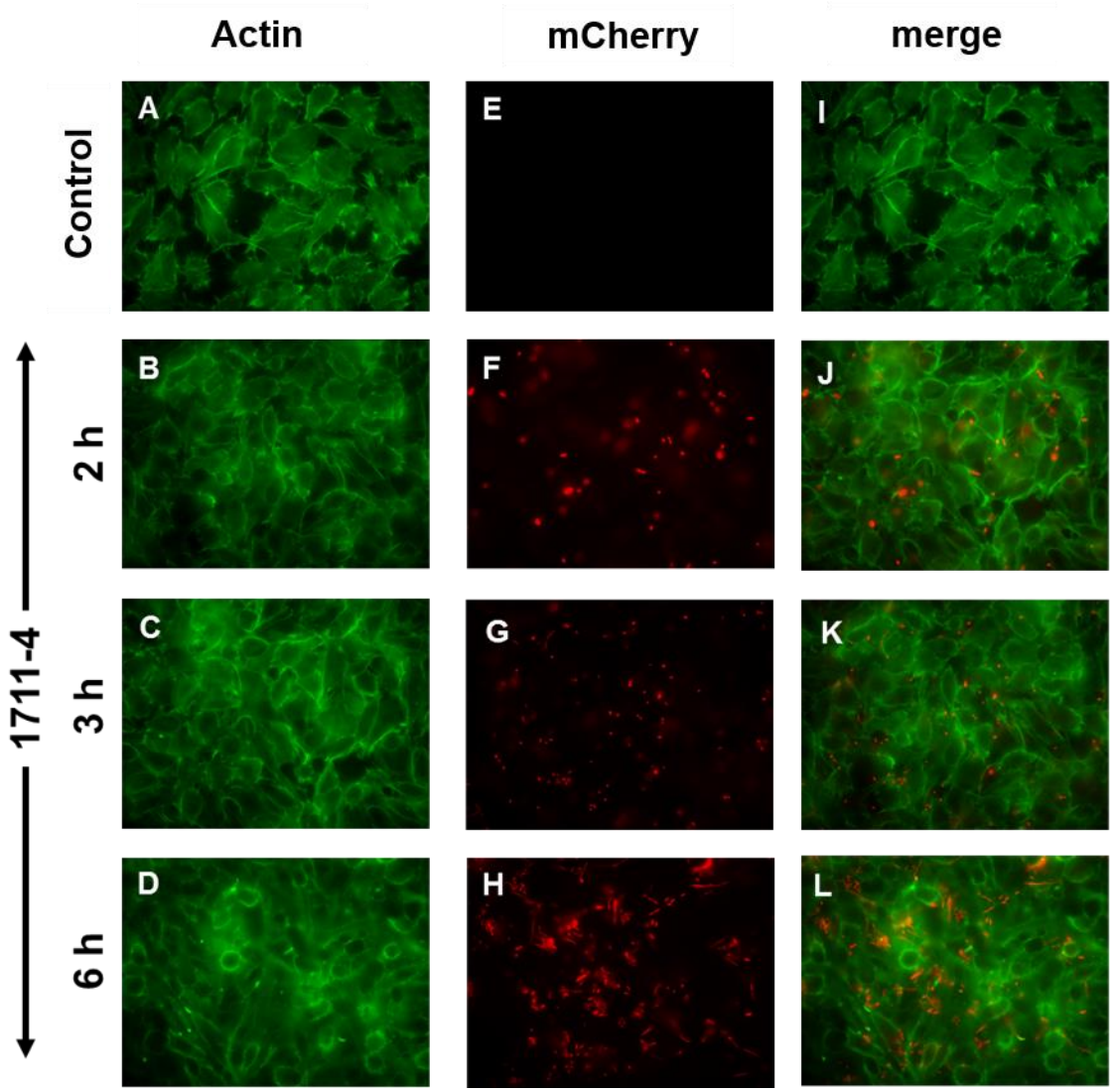


Figure 1 – Kinetic analyses of the interaction, invasion, and LEE gene and *fliC* expression during aEPEC 1711-4 incubation with polarized and differentiated Caco-2 cells. (A) kinetics of total interaction, showing that the numbers of bacteria interacting with Caco-2 cells in 3 and 6 hours were significantly higher than in 1.5 h, while no differences were observed between 3 and 6 h; (B) kinetics of invasion, showing that invasiveness could be observed only after 3 h, being significant after 6 h of interaction; (C) Relative expression of genes representing different LEE regions and flagella during the initial adherence and invasion processes; the expression of the *escJ* and *eae* genes increased from 1.5 h to 3 h and from 3 h

to 6 h; (D) kinetics of the bacterial intracellular persistence; no significant difference in bacterial counting was identified between 6 h and 24 h; (E) Relative gene expression of genes during the persistence; although expression of the *ler, escJ,* and *escN* genes increased, no significant difference was identified between 6 h and 24 h. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.0001$; for relative gene expression # $P \leq 0.05$ comparing 3 h and 6 h.



535 Figure 2 – Kinetic of pedestal formation of aEPEC 1711-4 in HeLa cells. The pedestal
536 formation was evaluated from 2 to 6 h of interaction. From 3 h to 6 h, the number of bacteria
537 interacting with HeLa cells and the number of pedestal structures per cell increased.

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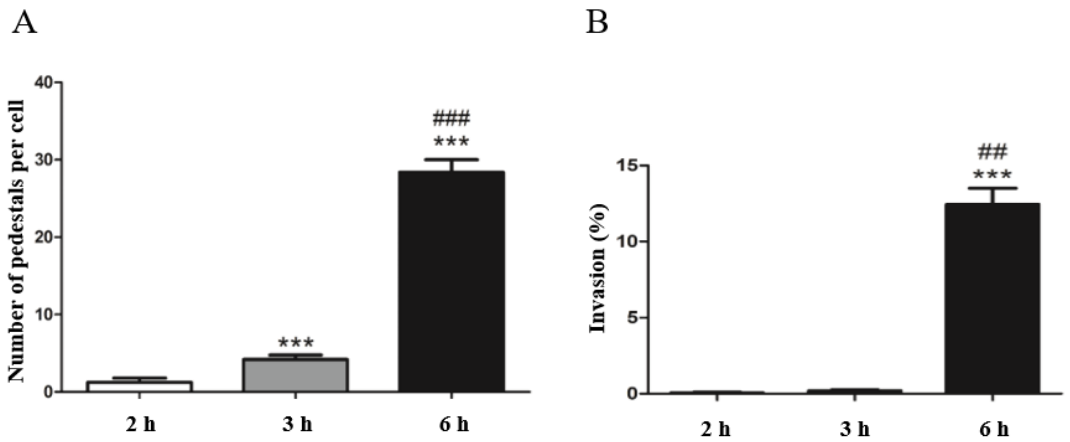
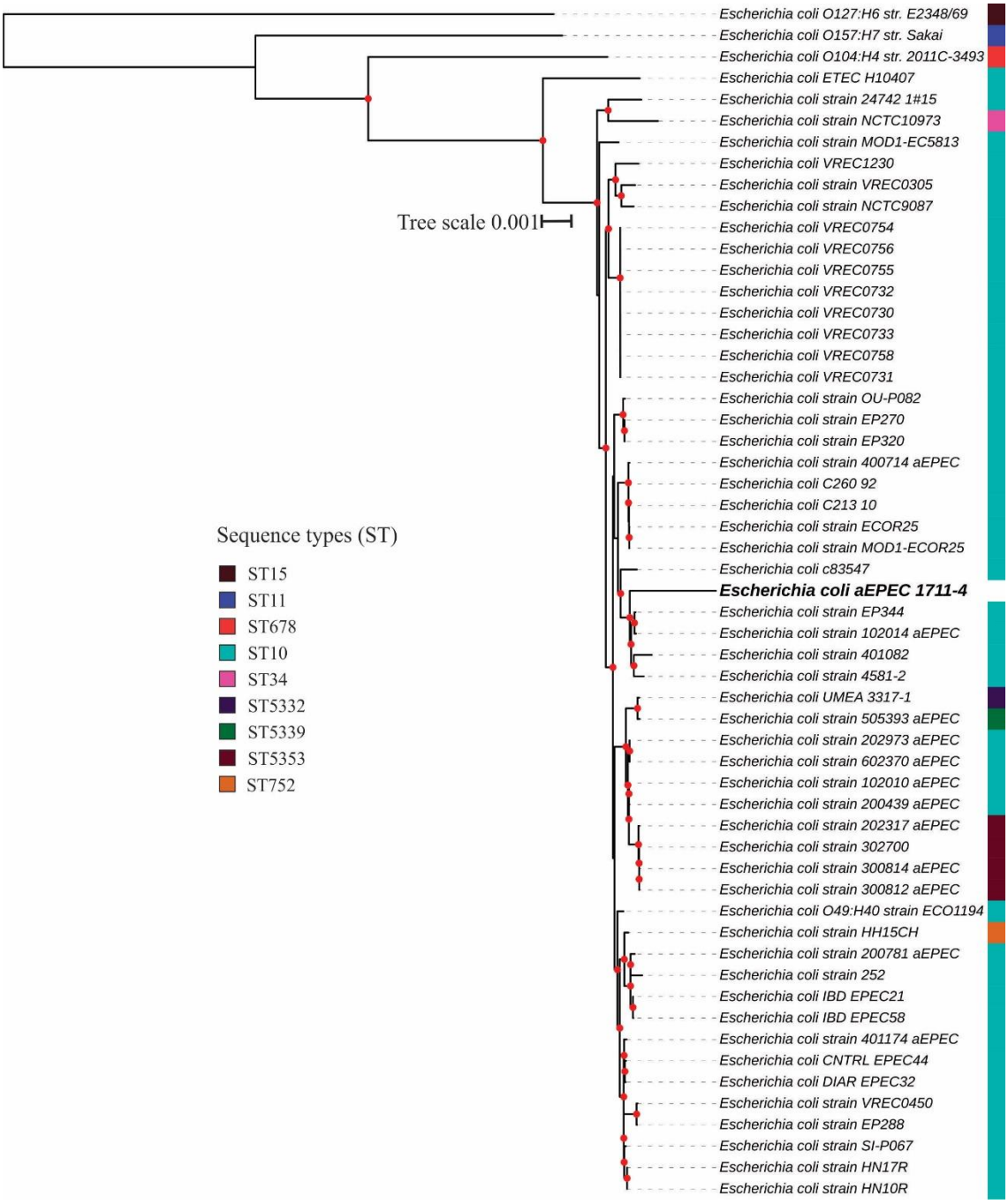


541

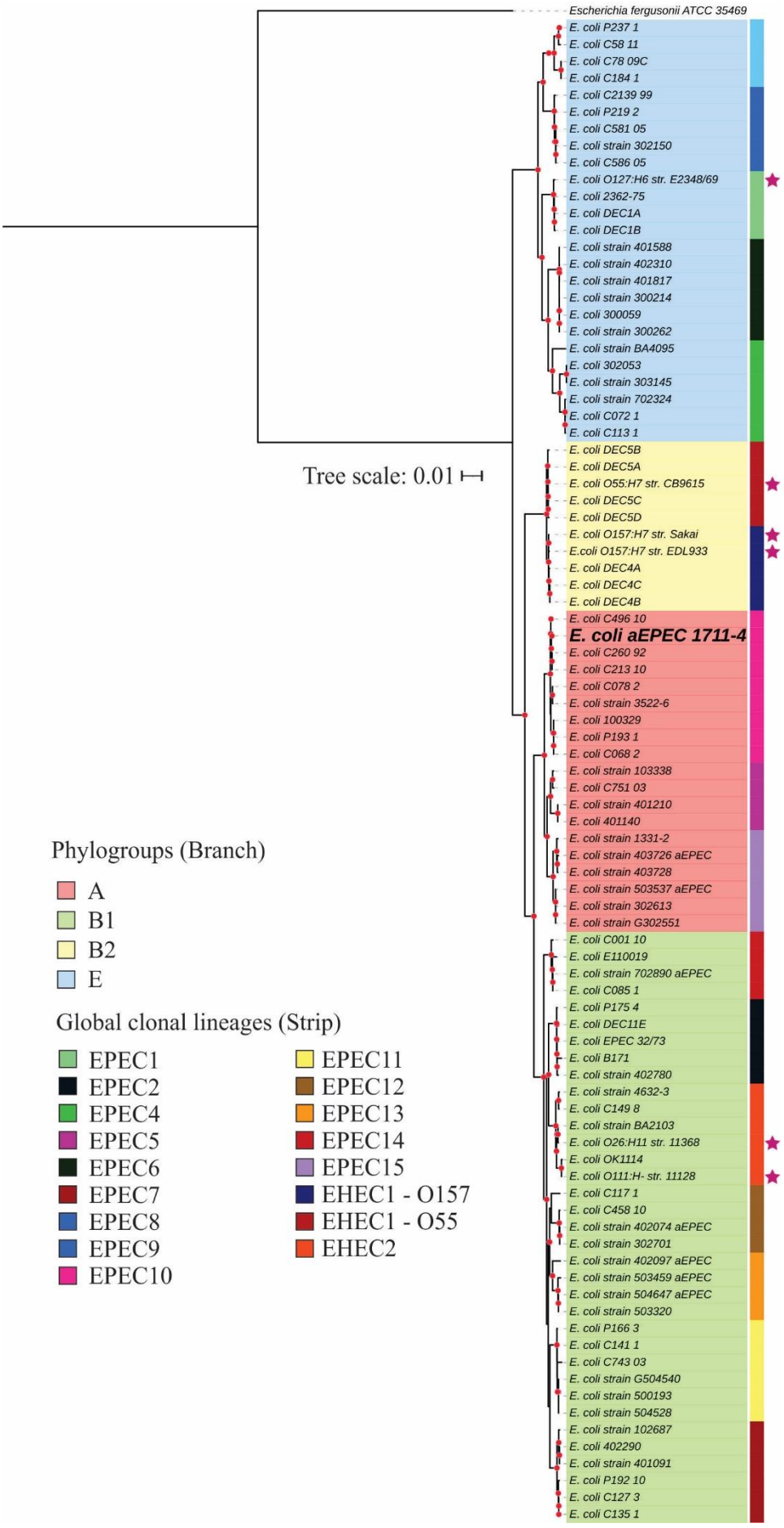
542 Figure 3 – Quantification of pedestal formation and invasion rates in HeLa cells. (A) The
543 number of pedestals per cell significantly increased from 2 h to 3 h and from 3 h to 6 h post-
544 infection. (B) The invasion rate was significantly high only after 6 hours post-infection. (*)
545 was used to express differences between 2 h against 3 h or 6 h, while (#) was used for
546 comparison between 3 h and 6 h. * $P \leq 0.05$, ** $P \leq 0.01$ *** $P \leq 0.0001$, ## $P \leq 0.01$, and
547 ### $P \leq 0.0001$.

548



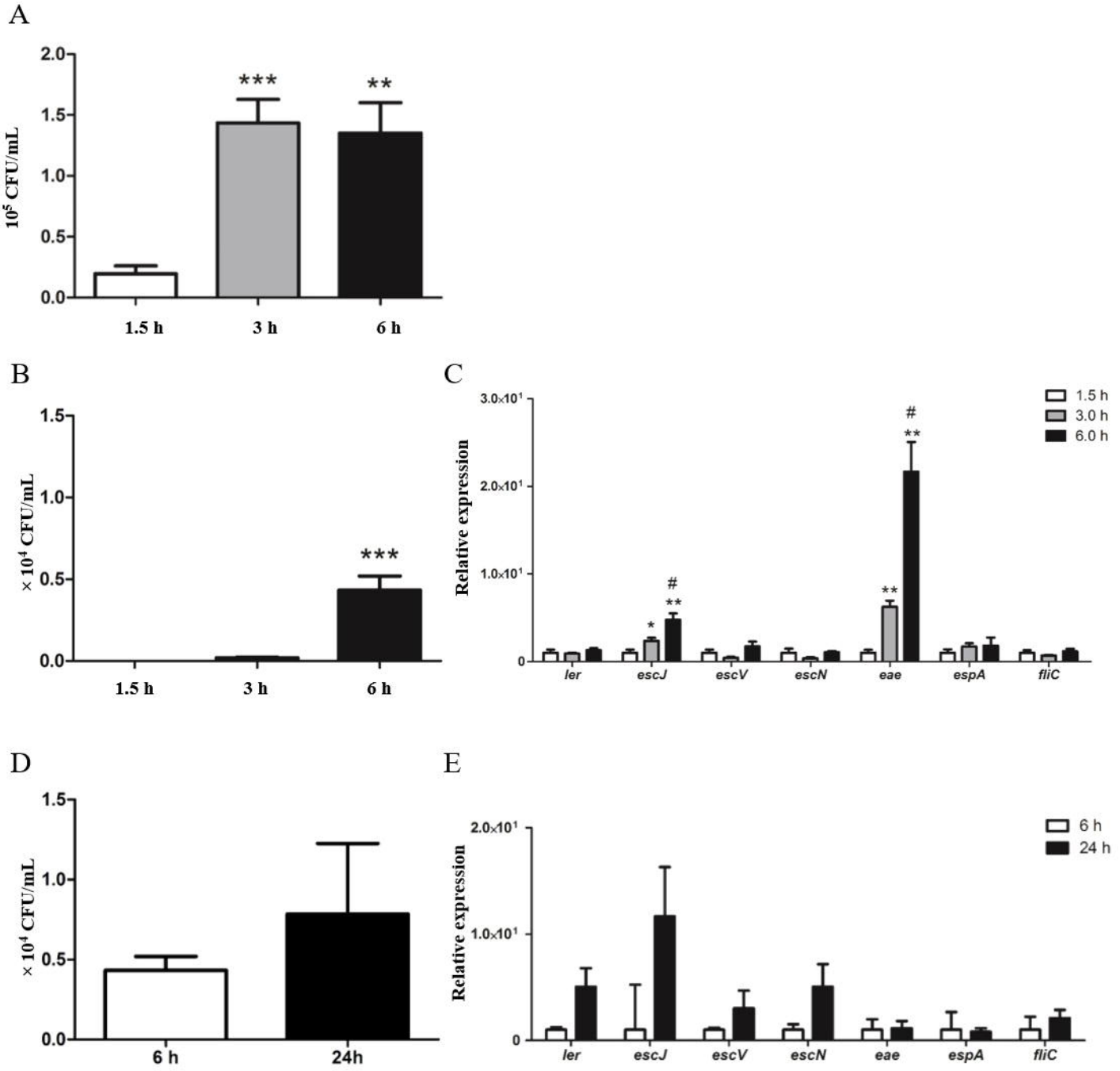
549

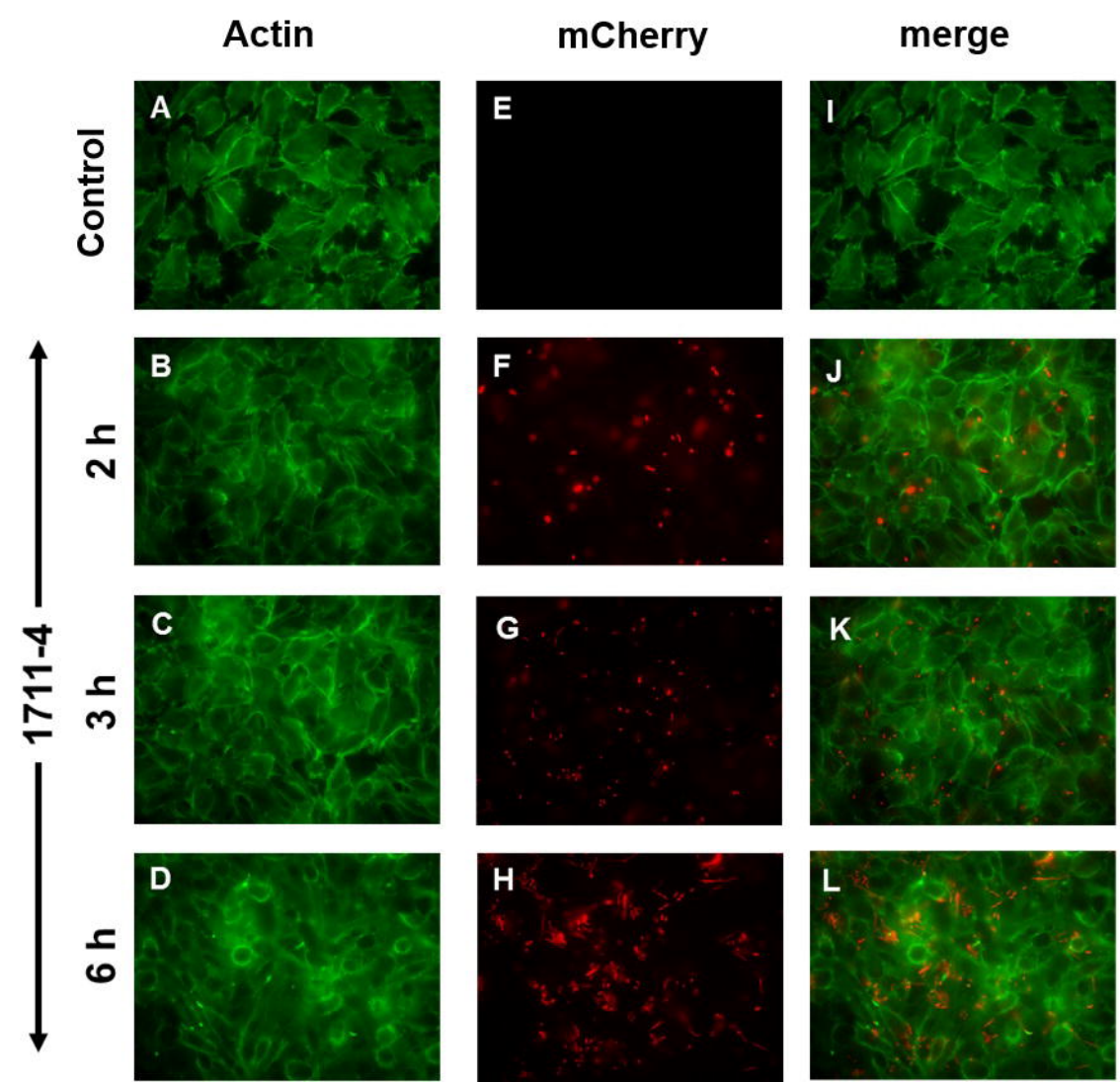
550 Figure 4 – aEPEC 1711-4 similar genomes codon tree. Red dots in the nodes represent
551 bootstrap ≥90. The *E. coli* prototype strains O127:H6 str. E2348/69 (ST15-B2), O157:H7 str.
552 Sakai (ST11-E), and O104:H4 str. 2011C-3493 (ST678-B1), and ETEC str. H10407 (ST10-
553 A) were used as outgroups.

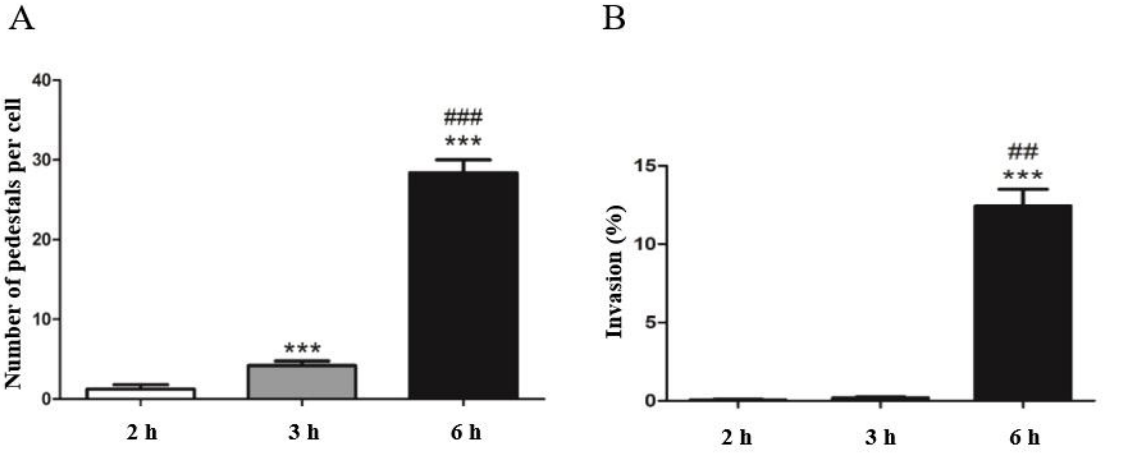


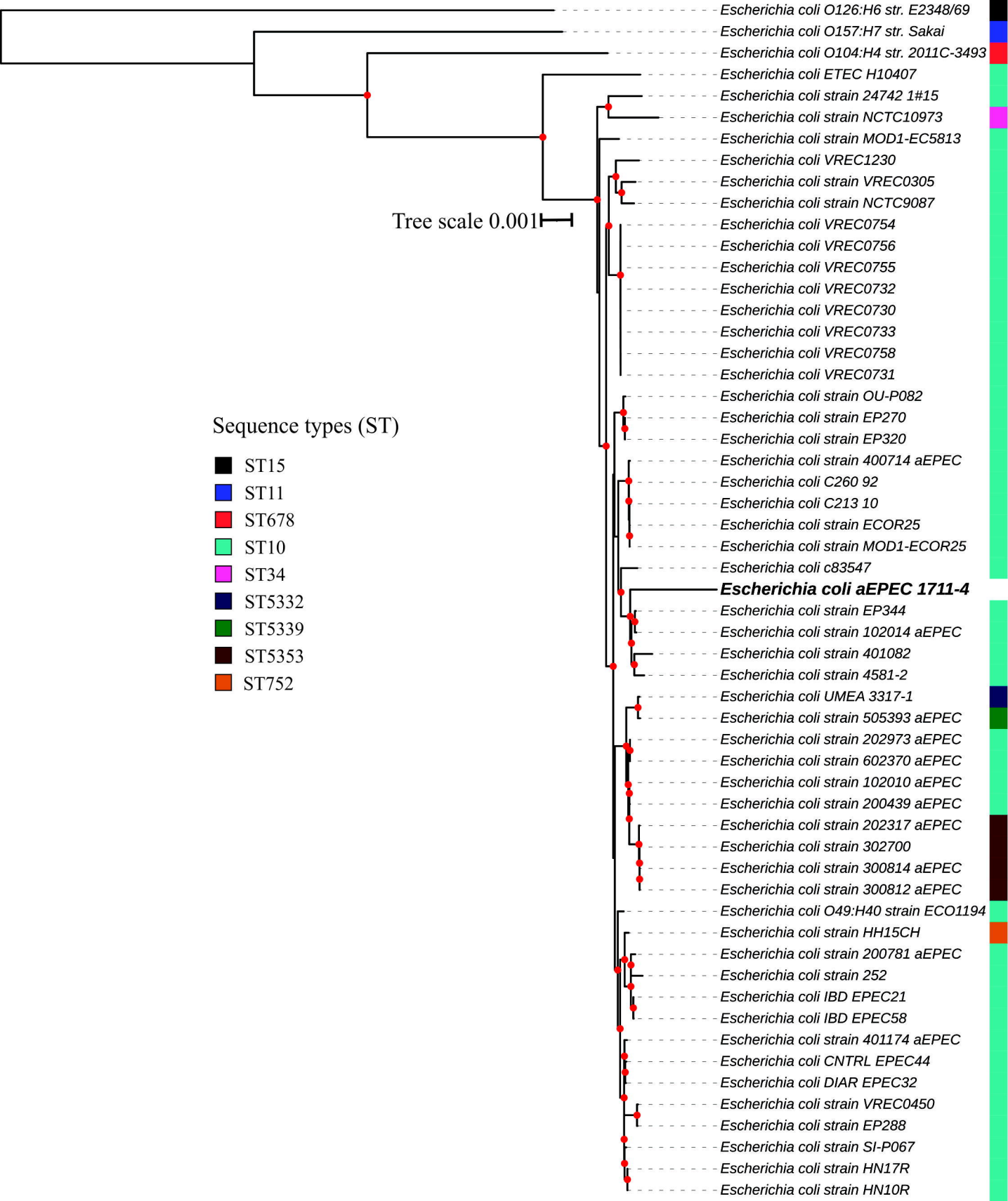
555 Figure 5 – Relationship of aEPEC 1711-4 and the EPEC and EHEC global lineages.
556 *Escherichia fergusonii* ATCC35469 was used as an outgroup to root the tree. A pink star in
557 the tree indicates EPEC and EHEC prototype or representative strains.

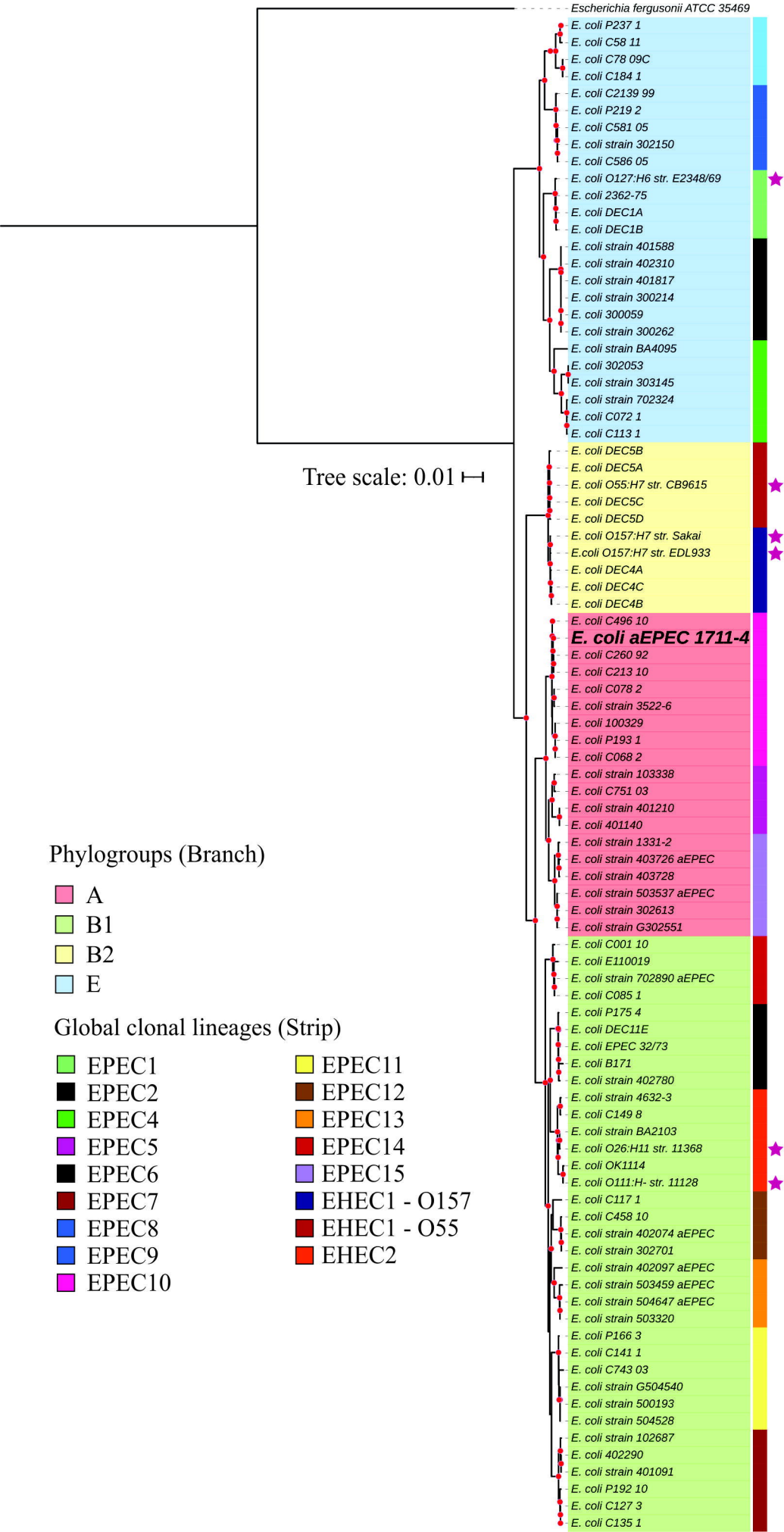
558


A
2.0
1.5
1.0
0.5
0.0
10^5 CFU/mL
***
**
1.5 h
3 h
6 h
B
1.5
1.0
0.5
0.0
× 10^4 CFU/mL
***
1.5 h
3 h
6 h
C
3.0×10^1
2.0×10^1
1.0×10^1
0
Relative expression
1.5 h
3.0 h
6.0 h
*
#
**
ler
escJ
escV
escN
eae
espA
fliC
D
1.5
1.0
0.5
0.0
× 10^4 CFU/mL
6 h
24h
E
2.0×10^1
1.0×10^1
0
Relative expression
6 h
24 h
ler
escJ
escV
escN
eae
espA
fliC


Actin
mCherry
merge
Control
1711-4
2 h
3 h
6 h
A
B
C
D
E
F
G
H
I
J
K
L


A
Number of pedestals per cell
40
30
20
10
0
###
***
***
2 h
3 h
6 h
B
Invasion (%)
15
10
5
0
##
***
2 h
3 h
6 h


Escherichia coli O126:H6 str. E2348/69
Escherichia coli O157:H7 str. Sakai
Escherichia coli O104:H4 str. 2011C-3493
Escherichia coli ETEC H10407
Escherichia coli strain 24742 1#15
Escherichia coli strain NCTC10973
Escherichia coli strain MOD1-EC5813
Escherichia coli VREC1230
Escherichia coli strain VREC0305
Escherichia coli strain NCTC9087
Tree scale 0.001
Escherichia coli VREC0754
Escherichia coli VREC0756
Escherichia coli VREC0755
Escherichia coli VREC0732
Escherichia coli VREC0730
Escherichia coli VREC0733
Escherichia coli VREC0758
Escherichia coli VREC0731
Escherichia coli strain OU-P082
Escherichia coli strain EP270
Escherichia coli strain EP320
Escherichia coli strain 400714 aEPEC
Escherichia coli C260 92
Escherichia coli C213 10
Escherichia coli strain ECOR25
Escherichia coli strain MOD1-ECOR25
Escherichia coli c83547
Escherichia coli aEPEC 1711-4
Escherichia coli strain EP344
Escherichia coli strain 102014 aEPEC
Escherichia coli strain 401082
Escherichia coli strain 4581-2
Escherichia coli UMEA 3317-1
Escherichia coli strain 505393 aEPEC
Escherichia coli strain 202973 aEPEC
Escherichia coli strain 602370 aEPEC
Escherichia coli strain 102010 aEPEC
Escherichia coli strain 200439 aEPEC
Escherichia coli strain 202317 aEPEC
Escherichia coli strain 302700
Escherichia coli strain 300814 aEPEC
Escherichia coli strain 300812 aEPEC
Escherichia coli O49:H40 strain ECO1194
Escherichia coli strain HH15CH
Escherichia coli strain 200781 aEPEC
Escherichia coli strain 252
Escherichia coli IBD EPEC21
Escherichia coli IBD EPEC58
Escherichia coli strain 401174 aEPEC
Escherichia coli CNTRL EPEC44
Escherichia coli DIAR EPEC32
Escherichia coli strain VREC0450
Escherichia coli strain EP288
Escherichia coli strain SI-P067
Escherichia coli strain HN17R
Escherichia coli strain HN10R
Sequence types (ST)
ST15
ST11
ST678
ST10
ST34
ST5332
ST5339
ST5353
ST752


Escherichia fergusonii ATCC 35469
E. coli P237 1
E. coli C58 11
E. coli C78 09C
E. coli C184 1
E. coli C2139 99
E. coli P219 2
E. coli C581 05
E. coli strain 302150
E. coli C586 05
E. coli O127:H6 str. E2348/69
E. coli 2362-75
E. coli DEC1A
E. coli DEC1B
E. coli strain 401588
E. coli strain 402310
E. coli strain 401817
E. coli strain 300214
E. coli 300059
E. coli strain 300262
E. coli strain BA4095
E. coli 302053
E. coli strain 303145
E. coli strain 702324
E. coli C072 1
E. coli C113 1
Tree scale: 0.01
E. coli DEC5B
E. coli DEC5A
E. coli O55:H7 str. CB9615
E. coli DEC5C
E. coli DEC5D
E. coli O157:H7 str. Sakai
E.coli O157:H7 str. EDL933
E. coli DEC4A
E. coli DEC4C
E. coli DEC4B
E. coli C496 10
E. coli aEPEC 1711-4
E. coli C260 92
E. coli C213 10
E. coli C078 2
E. coli strain 3522-6
E. coli 100329
E. coli P193 1
E. coli C068 2
E. coli strain 103338
E. coli C751 03
E. coli strain 401210
E. coli 401140
E. coli strain 1331-2
E. coli strain 403726 aEPEC
E. coli strain 403728
E. coli strain 503537 aEPEC
E. coli strain 302613
E. coli strain G302551
E. coli C001 10
E. coli E110019
E. coli strain 702890 aEPEC
E. coli C085 1
E. coli P175 4
E. coli DEC11E
E. coli EPEC 32/73
E. coli B171
E. coli strain 402780
E. coli strain 4632-3
E. coli C149 8
E. coli strain BA2103
E. coli O26:H11 str. 11368
E. coli OK1114
E. coli O111:H- str. 11128
E. coli C117 1
E. coli C458 10
E. coli strain 402074 aEPEC
E. coli strain 302701
E. coli strain 402097 aEPEC
E. coli strain 503459 aEPEC
E. coli strain 504647 aEPEC
E. coli strain 503320
E. coli P166 3
E. coli C141 1
E. coli C743 03
E. coli strain G504540
E. coli strain 500193
E. coli strain 504528
E. coli strain 102687
E. coli 402290
E. coli strain 401091
E. coli P192 10
E. coli C127 3
E. coli C135 1
Phylogroups (Branch)
A
B1
B2
E
Global clonal lineages (Strip)
EPEC1
EPEC2
EPEC4
EPEC5
EPEC6
EPEC7
EPEC8
EPEC9
EPEC10
EPEC11
EPEC12
EPEC13
EPEC14
EPEC15
EHEC1 - O157
EHEC1 - O55
EHEC2