# Emergence of enteroaggregative *Escherichia coli* within the ST131 lineage as a cause of extraintestinal infections

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Running title: Emergence of ESBL-producing EAEC ST131

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25 Word count: 250 abstract, 4849 main article.

## Abstract

*Escherichia coli* sequence type 131 (ST131) is a major cause of urinary and bloodstream infections and its association with extended-spectrum β-lactamases (ESBL) significantly complicates treatment. Most notorious is its rapidly expanding *H*30-Rx clade (named for containing allele 30 of the type-1 fimbrial adhesin gene *fimH* and extensive antimicrobial resistance), which appears to have emerged in the United States due in part due to the acquisition of the ESBL-encoding $bla_{\text{CTX-M-15}}$ gene and resistance to fluoroquinolones. However, non-*H*30 ST131 lineages with acquired CTX-M-type resistance genes also are emerging. Based on whole-genome analyses, we describe here the presence of an (*fimH*) *H*27 *E. coli* ST131 lineage that currently is causing an outbreak of community-acquired bacteremia and recurrent urinary tract infections (UTIs) in Denmark. This lineage has acquired both a virulence plasmid (pAA) that defines the enteroaggregative *E. coli* (EAEC) diarrheagenic pathotype and multiple genes associated with extraintestinal *E. coli* (ExPEC) that combined has made this particular ST131 lineage highly successful at colonizing its human host and cause recurrent UTI. Moreover, using a historic World Health Organization *E. coli* collection and publically available genome sequences, we identify a global *H*27 EAEC ST131 lineage dating back as far as 1998. Most *H*27 EAEC ST131 isolates harbor pAA or pAA-like plasmids, which analysis strongly imply was caused by a single ancestral acquisition. These findings illustrate the profound plasticity of this important pathogenic *E. coli H*27 lineage in general, and the genetic acquisitions of EAEC-specific virulence traits that likely confer an enhanced ability to cause intestinal colonization.


## Importance

The *E. coli* ST131 lineage is a notorious extraintestinal pathogen. A signature characteristic of ST131 is its ability to asymptomatically colonize the gastrointestinal tract and then opportunistically cause extraintestinal infections, such as cystitis, pyelonephritis and urosepsis. In this study, we report a novel ST131 sublineage that has acquired the enteroaggregative diarrheagenic phenotype, spread

51 across multiple continents and has been associated with multiple outbreaks of community-acquired
52 bloodstream infections in Denmark. The strain's ability to both cause diarrhea and colonize the
53 human gastrointestinal tract may facilitate its dissemination and establishment in the community,
54 whereas the strain's clonal nature may facilitate targeted control strategies, such as vaccination.

## Introduction

*Escherichia coli* sequence type 131 (ST131) is the dominant multidrug-resistant (MDR) extraintestinal pathogenic *E. coli* (ExPEC) lineage worldwide, causing a wide range of infections including bloodstream and urinary tract infections (BSIs/UTIs) (*1-3*). Its rise to global dominance and pathogenicity is thought to have been primed by sequential acquisition of virulence-associated genes followed by development of antibiotic resistance (*4, 5*). ST131 predominantly exhibits serotype O25:H4 and is closely associated with fluoroquinolone resistance and the production of the CTX-M-15 extended-spectrum β-lactamase (ESBL) (*2, 6*). The expansion of *E. coli* ST131 in the United States has been driven mainly by a single clade, designated *H*30 because of its tight association with allele 30 of the type-1 fimbrial adhesin gene, *fimH*. *H*30 has a prominent MDR-associated clade, *H*30R, which accounts for most fluoroquinolone resistance within ST131. *H*30R in turn has two main sub-clades: *H*30R, which accounts for almost all ST131-associated CTX-M-14 and CTX-M-27 production (although most members are ESBL-negative), and *H*30Rx, which accounts for almost all ST131-associated CTX-M-15 production (*6*). In addition to *H*30, other distinct clades of ST131 are also circulating worldwide, most commonly associated to the 22 and 41 *fimH* alleles. While these non-*H*30 allelic variants are normally not associated with carriage of CTX-M-genes, cases of $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-27}}$ acquisition by ST131 lineages carrying *fimH*22 or –*H*41 have been reported (*4, 7*).

ST131 isolates typically carry multiple ExPEC-associated virulence genes encoding adhesins, toxins, and siderophores, whereas virulence genes typical of diarrheagenic *E. coli* (DEC) rarely have been reported (*8, 9*). However, we recently surveyed an international World Health Organization (WHO) collection of historic *E. coli* for ST131 O25 isolates, assessing them for temporal trends of antibiotic resistance and virulence traits. Among a total of 128 ST131 isolates we found 12 (9%) that fulfilled molecular criteria for the enteroaggregative *E. coli* (EAEC) pathotype (*10*). Pulsed-field gel electrophoresis (PFGE) analysis revealed a cluster comprising seven of these

EAEC isolates. Of these, six – including two urine isolates from patients with UTI, three fecal isolates from patients with diarrhea, and one lower respiratory tract isolate (associated symptoms unknown) – were from Danish patients (1998-2000), supporting the occurrence of an unrecognized EAEC ST131-associated outbreak of UTI and possibly also diarrhea in this time period in Denmark (*10*).

EAEC strains cause endemic diarrheal illness in developing countries and foodborne outbreaks in developed countries, and have been associated with extraintestinal infections (*11*). This pathotype gained particular attention following a major foodborne outbreak in Germany in 2011 that was caused by a Shiga toxin (Stx)-producing O104:H4 EAEC strain and resulted in 3,842 confirmed cases and 54 deaths (*12*). Additionally, a worryingly high prevalence of multidrug resistance among EAEC strains has been reported, and reports of CTX-M-type ESBL-producing EAEC have appeared recently from around the world (*13-16*). EAEC pathogenesis involves adherence to human intestinal mucosa by virtue of aggregative adherence fimbriae (AAF) and subsequent biofilm formation. The AAF are encoded on large plasmids designated pAA that also encodes a suite of other EAEC virulence factors, including AggR, a global regulator of EAEC virulence; dispersin, required for proper dispersal of AAFs on the bacterial surface; the AatPABCD transporter system, which mediates dispersin secretion; and Aar, a recently described negative regulator of AggR (*17, 18*).

To understand the emergence and underlying genetic acquisitions leading to ESBL-producing EAEC ST131, we investigated the relatedness of a large collection of temporally and spatially diverse ST131 isolates. This investigation revealed the emergence of a global *H*27 lineage of EAEC linked to a single acquisition of the pAA plasmid that likely improved the strain's ability to persistently colonize its human host.

## Results

**Identification of historic Danish EAEC ST131**. Our previous PFGE-based analysis of ST131 O25 isolates within the WHO *E. coli* collection identified an apparently unrecognized outbreak of UTI, and possibly also diarrhea, in Denmark in 1998-2000 that was caused by a group of seemingly similar EAEC isolates (*10*). To better estimate these isolates' relatedness and to correct for recombination (*6*), all 128 ST131 isolates from the WHO collection were subjected to whole-genome sequencing. The *in silico* analyses regarding the genotypic features of the isolates revealed that the most frequent *fimH* alleles were *H*30 (51%, n=65), *H*22 (34%, n=44) and *H*27 (7%, n=9). Most of the isolates harbored multiple antibiotic resistance genes. The most common ESBL gene was $bla_{\text{CTX-M-15}}$ (37%, n=47), which occurred almost exclusively within the *H*30 subgroup (98%, 46/47). See **Suppl. Table 1.**

To ensure that only high-quality genome data sets were included in our SNP analysis, we excluded the genome data for seven (5%) of the 128 ST131 isolates due to low sequencing depth. In addition, we included genome sequences from a published collection of human clinical ST131 *E. coli* (n = 93) collected in the USA and Germany between 2010 and 2012 (*6*). Across these genomes, 11,529 SNPs were identified within ~38.4% of the JJ1886 genome that was conserved across all isolates. After purging of recombinant regions, 4,241 SNPs were used to infer relationships between the isolates.

The SNP-based phylogeny showed distinct overall clustering of isolates in accordance to their *fimH* alleles, with all 116 isolates carrying the *fimH*30 allele clustering as a monophyletic clade (**Suppl. Fig. 1**). Likewise, all 10 isolates carrying the *fimH*27 allele clustered within a single clade which – like the *H*30 clade – appeared to descend from an ancestral *H*22 group. Of these *fimH*27-carrying isolates, eight – all from the WHO collection – qualified molecularly as EAEC based on presence of ≥1 of the EAEC-associated putative virulence genes *aggR*, *aatA*, and *aaiC (19)*. On the basis of previous PFGE data (*10*), seven of these eight EAEC isolates had formed a cluster, whereas the remaining isolate (C796-00) appeared quite distinct from the others. By contrast, here all

eight isolates clustered together, with C796-00 being most closely related to three of the other seven EAEC isolates (**Suppl. Fig. 1**). Included in this cluster of *fimH*27-carrying EAEC isolates were C86-04, a fecal isolate from Vietnam (2004), and seven Danish isolates, including two urine isolates, one lower respiratory tract isolate, and four fecal isolates from patients with diarrhea, suggesting that this lineage was able to cause both extraintestinal infections and diarrhea.

Unlike the PFGE analysis, the SNP-based analysis additionally revealed a distinct cluster of isolates located within the *H*22 group consisting of the remaining four EAEC isolates from the WHO collection (**Suppl. Fig. 1**). Three of the isolates carried *fimH*22, whereas one isolate carried *fimH*298, which differs from *fimH*22 by only a single nucleotide. All four isolates were from urine samples collected within a 10-month period in 1998, from elderly patients admitted to four different departments of the same hospital in the Capital Region in Denmark, suggesting an unrecognized nosocomial UTI outbreak. With the exception of Vietnamese isolate C86-04, all 11 EAEC isolates were recovered from Danish patients. The Vietnamese isolate carried $bla_{\text{CTX-M-27}}$, whereas no ESBL genes were present in the other 11 EAEC isolates. (**Suppl. Table 1**).

**EAEC virulence genes in historic Danish EAEC ST131.** The four clustered EAEC urine isolates that carried *fimH*22 or *fimH*298 all harbored the *aggDCBA* gene cluster encoding the AAF/I variant, whereas seven of the eight clustered *fimH27*-carrying EAEC isolates of diverse sources all harbored *agg5DCBA* encoding the recently described AAF/V variant (*20*). The remaining *H*27 isolate, C1883-99, lacked the *agg5A* gene (**Suppl. Table 2**). All but two of the 12 EAEC isolates contained genes encoding AggR, Aar, dispersin (*aap*), and the AatPABCD transporter system, plus two additional AggR-regulated open reading frames (ORFs), ORF3 and ORF4, that are assumed to play a role in isoprenoid biosynthesis (*21*). The remaining two isolates, C1883-99 (*H*27) and C167-00 (*H*22), both lacked most of these EAEC-specific genes, implying partial deletion of the pAA plasmid in isolates from both these *fimH*-associated clades.

**Invasive EAEC ST131 isolates with *bla*$_{\text{CTX-M-101}}$ obtained from Danish patients**. To determine whether EAEC ST131 strains were present among contemporary Danish patients, we searched for EAEC-specific virulence genes within a recently published collection of 552 whole-genome sequenced ESBL-producing *E. coli* (ESBL-*Ec*) isolates obtained from patients with BSIs in Denmark between 2014 and 2015 (*22*). ST131 accounted for 50% (n = 258) of the isolates (*22*). Among these, 25 isolates harbored the genes encoding AAF/V, AggR, Aar, Aap, AatPABCD, and ORF3/4 (**Suppl. Table 2**). All 25 carried *fimH*27 and *bla*$_{\text{CTX-M-101}}$. In total, 27 isolates in the collection were *bla*$_{\text{CTX-M-101}}$-positive (*10*), of which thus 93% qualified molecularly as EAEC. The EAEC pathotype did not occur in conjunction with any other ESBL-encoding gene; hence, the association between *bla*$_{\text{CTX-M-101}}$ and the EAEC pathotype was highly significant ($\chi^2$, $p<0.0001$), suggesting that the resistance gene may be co-localized with EAEC-specific virulence genes on the pAA plasmid. As shown by Roer *et al.*, the 27 ST131 strains with *bla*$_{\text{CTX-M-101}}$ formed a distinct cluster with nine or fewer SNP differences, supporting a recent emergence (*22*).

To assess whether the same ESBL-EAEC ST131 strain could be detected in the urine of the patients from whom the positive blood samples were collected, urine isolates from eight of the 258 patients with an ESBL-EAEC ST131 BSI were available and sequenced. Notably, all of these patients had presented with UTI either prior to, concurrent with, or subsequent to the time of blood sampling. Thus, some patients provided more than one urine sample. Specifically, one patient presented 19 days after an initial bacteremia episode with both recurrent bacteremia and UTI, all involving the ESBL-EAEC ST131 strain, and four other patients had recurrent same-strain UTI diagnosed from three weeks to eight months after their initial UTI episode. SNP analysis demonstrated that the corresponding blood and urine isolates were nearly identical (i.e., differed by ≤6 SNPs), whereas different pairs were separated by zero to 11 SNPs. Of the eight pairs, five clustered distinctively in the phylogeny thus strongly implying their relatedness. For the remaining

three pairs of blood and urine isolates, the data were inconclusive (**Suppl. Fig. 2**). These findings suggest strongly that the ESBL-EAEC ST131 strains were capable of long-term persistence in these hosts and/or their immediate environment and of causing repeated episodes of UTI and bacteremia.

**pAA plasmid characterization in ST131 isolates.** To characterize in detail the pAA plasmids present in the ESBL-EAEC ST131 strains, we applied MinION sequencing (Oxford Nanopore Technologies) to obtain the complete plasmid sequence for one such plasmid, which we designated pAA-ST131, from a single representative Danish *E. coli* isolate (ESBL20150001, Sequence Read Archive (SRA) ID: DF215RRW). The complete plasmid sequence was 142,646 bp in length, with an average G+C content of 48.7% (**Fig. 1,** GenBank accession no. KY706108). A total of 197 open reading frames (ORFs) were predicted and annotated, of which 142 were functionally assigned. Two replicons were identified, RepFII and RepFIB, with the multireplicon F plasmid FAB formula of F1:A-:B33.

pAA-ST131 contained several genes associated with plasmid stability, including *parA*, *parM*, and *stbB*, plus three toxin-antitoxin (TA)-based addiction systems: *ccdAB*, *vagCD*, and *relBE* (**Fig. 1**). Furthermore, it harbored a 33 kb complete *tra* region encoding transfer components (24 *tra* genes, 8 *trb* genes, and *finO*), implying that the plasmid may be conjugally transferable. It also contained genes encoding putative transmembrane proteins and proteins involved in catabolism and metabolism, plus several integrated mobile elements. It contained all the EAEC virulence factor genes already identified by Illumina sequencing (as expected), but no additional known virulence factor genes or antibiotic resistance genes including $bla_{\text{CTX-M-101}}$. Because isolate ESBL20150001 contains no additional plasmids (**Suppl. Fig. 3**), we conclude that $bla_{\text{CTX-M-101}}$ is chromosomally located.

A BLAST comparison of pAA-ST131 with five complete pAA plasmids (Genbank accession IDs NC_018666 (O104:H4), NC_011752 (55989), FN554767 (042) and NC_008460

(DIJ1) and SRA ID SRA055981 (226)) showed that all these plasmids shared common features (**Fig. 1**), mostly corresponding to EAEC virulence genes. The entire genomic sequences were not available for all corresponding EAEC isolates but they belonged to at least three STs (ST40, ST414, and ST678). However, they also varied substantially for genetic content, as described previously for *E. coli* virulence plasmids (*23*). Specifically, regions containing putative metabolic, catabolic, and transmembrane proteins were unique to pAA-ST131 (**Fig. 1**). Additionally, although (like pAA-ST131) four of the five reference pAA plasmids also contained the RepFIB and/or the RepFIIA replicon, only three contained both. Moreover, only two contained the *tra* region.

**Global emergence of EAEC ST131 isolates carrying *fimH*27.** To assess the global extent of EAEC ST131 isolates, we screened for *aggR* (the global regulator of EAEC virulence) among all >3,500 *E. coli* ST131 genomes (as of November 15$^{th}$ 2017) available in EnteroBase (http://enterobase.warwick.ac.uk), a database with >100,000 genomic assemblies of enteric bacteria, including *E. coli*. Disregarding the *aggR*-positive, $bla_{\text{CTX-M-101}}$-containing Danish EAEC isolates, we identified another 25 international *aggR*-positive isolates, which carried the following *fimH* alleles (no. of isolates, % of 25): *fimH*27 (18, 66%), *fimH*22 (one, 4%), *fimH*298 (two, 8%), *fimH*30 (one, 4%), *fim*H5 (two, 8%), and *fim*H54-like (one, 4%) (**Suppl. Table 2**). The *aggR* gene occurred in 18 (38%) of the 47 other *fimH*27-carrying ST131 isolates in EnteroBase.

To determine whether the 12 EAEC ST131 isolates from the WHO collection were clonally related to other available EAEC ST131 isolates, a SNP-based phylogeny was constructed using 1) all 121 ST131 isolates from the WHO collection (*10*); 2) 93 German/US ST131 isolates from 2010-2012 (*6*); 3) the 27 Danish $bla_{\text{CTX-M-101}}$-containing ST131 isolates from 2014-2015 (*22*); and 4) 46 international *fimH*27-carrying ST131 isolates from Enterobase. Focusing first on isolates within the *H*22 clade (**Fig 2A**), WHO collection EAEC isolate C180-00, carrying *fimH298*, clustered together with two *fimH*298 EAEC EnteroBase isolates (SRA IDs SRR2970775 and SRR2970774)

229 from Cambodia (2009-2010). In contrast, the three WHO collection EAEC isolates carrying *fimH*22
230 were not closely related to the only *fimH22* ST131 isolate from EnteroBase.

231 In the rooted phylogeny, most EAEC isolates nested within the *H*27 clade (**Fig. 2A**).
232 To improve resolution, this clade's 79 isolates (76 with *fimH27*, two with *fimH5*, one with *fimH54*)
233 were analyzed separately. The 27 $bla_{\text{CTX-M-101}}$-containing Danish EAEC isolates formed a distinct
234 cluster with very short branches indicating a recent emergence (**Fig. 2B**). By contrast, none of the
235 *fimH*27-carrying international (i.e., non-Danish) isolates clustered either with the Danish isolates or
236 with one another, suggesting that the Danish outbreak was confined to Denmark, and that no other
237 focal outbreaks were captured. The WHO collection's *H*27 clade EAEC isolates were intermingled
238 with isolates from the UK, Thailand, and Canada (**Fig. 2B**), suggesting global spread of a common-
239 ancestry strain.

240 To determine the mosaicism of the EAEC-specific virulence genes among *fimH*27-
241 carrying ST131 isolates, sequence reads from all 79 *H*27 clade isolates were mapped against pAA-
242 ST131 from ESBL20150001. Intriguingly, pAA-ST131 was highly conserved among the *H*27 EAEC
243 isolates (**Fig. 2B)**. To validate the *in silico* results, plasmid gel profiling was done for representative
244 *fimH*27-carrying $bla_{\text{CTX-M-101}}$-containing EAEC isolates and EAEC isolates from the WHO
245 collection. This confirmed that all but three of the ten tested isolates contained a single plasmid of
246 conserved size (~140 kb). When mapping the sequence reads to pAA-ST131 these all showed 99-
247 100% coverage. The three exceptions included WHO isolate C1883-99, which harbored a slightly
248 smaller plasmid, and isolates ESBL20150196 and ESBL20150300 (both obtained from Danish
249 patients in 2015), each of which harbored a single 30-35-kb plasmid that, based on *in silico* mapping,
250 was the result of a single major deletion that left only the RepFIB replicon (**Suppl. Fig. 3)**.

251 Next, we performed a SNP-based analysis of pAA-ST131 across all 57 *H*27 isolates
252 with more than 70% coverage of the plasmid, with the four *H*22 clade EAEC isolates from the WHO
253 collection used as an outgroup. This identified 197 SNPs within the ~25% of pAA-ST131 that was

conserved across all isolates. Phylogenetic analysis based on these SNPs showed that pAA-ST131 is highly conserved among the *H*27 isolates (including the three with a truncated version of the plasmid), but differs considerably between the *H*27 and non-*H*27 isolates (**Suppl. Fig. 4**), strongly suggesting a single ancestral acquisition of the plasmid within the *H*27 clade, with subsequent partial deletions (to give the observed smaller variants).

Finally, we performed a BLAST analysis to screen the *H*27 isolates for classical ExPEC virulence genes (**Suppl. Table 3**). All *H*27 isolates carried the *sfa*, *iutA*, and *kpsM* II genes, which qualified them molecularly as ExPEC (*24*). In addition, they all contained the *chuA*, *fyuA*, and *yfcV* genes, which moreover qualified them molecularly as uropathogenic *E. coli* (UPEC) (*25*).

## Discussion

*E. coli* ST131 is a notorious MDR ExPEC lineage associated with both UTI and BSI (*1*). Little is known about which genes (whether promoting virulence or other phenotypes) make this clonal lineage so successful. ST131 strains have been shown to exhibit high levels of genomic plasticity including frequent recombination and plasmid flux, particularly involving IncF-type plasmids, facilitating spread of antibiotic resistance and virulence genes (*4, 26*). Indeed, ST131 lineages exhibit extensive variation with regard to acquired virulence genes (*2, 27*). Many typical ExPEC-associated virulence factors – including P fimbriae, hemolysins, and factors conferring increased serum survival and iron uptake – have been identified in ST131 isolates (*9, 10*). In contrast, virulence traits associated with diarrheagenic *E. coli* (DEC) pathotypes have thus far largely been absent from ST131 isolates *(10)*.

In developed countries, EAEC is known mostly as a cause of self-limiting diarrhea of mild to moderate severity. Indeed, long-term carriage of EAEC has been suggested to lead to colonization rather than infection (*11*). By contrast, in developing countries EAEC is a leading cause of childhood diarrhea (*11, 28*). The pathogenic potential of EAEC is underscored by its ability to

cause major foodborne outbreaks of diarrheal disease (*19, 29, 30*). Intriguingly, recent studies have associated EAEC with UTI, suggesting that what classically has been regarded as a DEC pathotype may also qualify as ExPEC and cause both diarrhea and UTI (*31-33*).

Like other DEC pathotypes, EAEC has been shown to encompass diverse genetic lineages, reflecting a high level of phylogenetic heterogeneity (*16, 34*). Although until recently no extraintestinal EAEC ST131 had been documented, recent reports have described the occurrence in certain ST131 subclonal lineages of EAEC-specific traits in geographically distinct areas (*10, 13, 16*). ESBL-producing ST38 and other ST types of various serotypes have been indicated as emerging hybrid strains of UPEC and EAEC involved primarily in UTIs from Germany, the Netherlands and the United Kingdom (*35*). In a study of fecal and urine isolates from Danish patients in 1998-2000, we made the novel observation of EAEC ST131 of serotype O25 (*10*). Subsequently, another study documented the emergence of a $bla_{\text{CTX-M-14}}$-containing EAEC ST131 O25:H4 strain in stool samples of diarrheic patients in Japan from 2003 onwards (*13*).

Our previous PFGE analysis of Danish ST131 O25 *E. coli* isolates from the WHO collection demonstrated that seven of the 12 identified EAEC strains were highly similar and appeared to have been part of an unrecognized UTI and diarrhea outbreak in Denmark in 1998 to 2000 (*10*). Although the genomic approach we used here to analyze the same isolates confirmed the suspected outbreak, it also yielded certain substantially different conclusions from the PFGE analysis, including identification of a second distinct cluster of EAEC urine isolates. These discrepancies between WGS SNP-based analysis and PFGE analysis among ST131 isolates correspond with previous findings (6).

Here we also document the current presence of a novel ESBL-EAEC ST131 sublineage as a cause of bacteremia in patients admitted to Danish hospitals. The isolates, as collected across Denmark over a 16-month period in 2014-2015, all carried $bla_{\text{CTX-M-101}}$. Notably, to date the only other reports of CTX-M-101-positive *E. coli* are from China (*36-38*). Roer *et al*. used a genomic

304 approach to establish that the Danish ESBL EAEC isolates were highly clonal, strongly suggesting a
305 recent common source (*22*). Here, by analyzing sequential urine isolates from eight of the patients
306 with the Danish outbreak-associated ESBL-EAEC ST131 bacteremia, we found that five patients had
307 recurrent UTI caused by the outbreak strain. The time of paired urine sampling ranged from one
308 week before to eight months following the initial blood sampling. The Danish ESBL-EAEC ST131
309 outbreak strain thus seems capable of persistently colonizing patients, resulting in occasional clinical
310 manifestations. Less likely, the patients may have been continuously exposed from an external
311 source.

312 Using the EnteroBase collection of ST131 genome sequences, we identified the global
313 presence of *fimH*27-carrying EAEC ST131 isolates. These isolates originated from Africa, Asia,
314 Europe and North America, and together with the Danish EAEC ST131 outbreak isolates spanned
315 more than two decades. Strikingly, all the EAEC ST131 strains studied here proved to be clonal, i.e.,
316 to share a common ancestor. Moreover, they share a common virulence plasmid, designated pAA-
317 ST131, which appears to have been acquired once in the *H*27 clade and transmitted vertically,
318 although it has undergone recombination and deletion events in some sublineages. MinION-based
319 sequencing of this plasmid in a representative ESBL-EAEC strain identified an array of classical
320 plasmid-encoded EAEC-defining virulence genes, including those encoding AAFs and the global
321 regulator of virulence AggR.

322 The acquisition of pAA plasmids by *E. coli* ST131 is interesting, considering that the
323 ST131 lineage is thought of as a host generalist (*39*), whereas EAEC appears to be highly adapted to
324 humans, without a natural animal reservoir (*40, 41*). However, the EAEC ST131 lineage described
325 here is reminiscent of the UTI outbreak in Copenhagen in 1991 caused by an *E. coli* O78:H10 clonal
326 group in that both fulfilled the molecular criteria for EAEC and contained multiple ExPEC virulence
327 genes (*32*). That the O78:H10 outbreak strains' EAEC-associated virulence factors were found to
328 increase uropathogenicity (*33*) suggests that this may be true also for EAEC ST131 strains. The

329 O78:H10 EAEC outbreak strain, which never was found outside the Capital Region of Denmark and
330 was not known to have caused BSI. In contrast, ST131 is a highly successful pandemic clonal group,
331 which makes the potential of future outbreaks of UTI and bacteremia caused by this novel strain, or
332 other EAEC ST131 lineages, a cause for serious concern.

333 Despite the genetic evidence that all 25 $bla_{\text{CTX-M-101}}$-containing Danish EAEC isolates
334 were derived from a common source, based on the limited available epidemiological data we have
335 been unable to establish a patient link or explain the spread of the lineage across regions. UTI is
336 often caused by enteric *E. coli* that enter the urinary tract via the fecal-perineal-urethral route, and in
337 some instances may have as their proximate external source food products or animals (*42*).
338 Interestingly, the gender ratio among the 25 present cases (68% females, 32% males), differs
339 significantly ($p$ = 0.01) from that across the entire collection of 552 ESBL-producing ST131
340 bloodstream isolates from Danish hospitals in 2014-2015 (42% females, 58% males) (*22*). A similar
341 overrepresentation of women was observed among cases during the 2011 multinational European
342 outbreak caused by a novel multi-pathotype, Shiga-toxin-producing EAEC O104:H4 strain (*43*).
343 Bean sprouts were found the most likely vehicle of infection, and the high proportion of female cases
344 in that study was thought to be driven by the tendency of women to be more health conscious and
345 perhaps suggestive of a food-related source for the Danish ESBL EAEC ST131 outbreak. Further
346 screening, e.g. of fecal and urine samples from past and current patients who present with diarrhea or
347 UTI, are warranted to determine the true clinical impact of the ESBL EAEC ST131 strain and to
348 provide the demographic and epidemiological data needed to identify potential sources.

349 In conclusion, we hypothesize that acquisition of the pAA plasmid made this *H*27
350 ESBL EAEC ST131 lineage highly successful at persistently colonizing patients, thereby allowing it
351 to occasionally cause UTIs and diarrhea. The presence of multiple ExPEC virulence factors –
352 including P fimbriae, α-hemolysin, and Sat – in turn facilitates dissemination from the urinary tract
353 to the bloodstream. Furthermore, we have demonstrated the ability of a non-*H*30 ST131 lineage to

acquire EAEC-specific pAA virulence plasmids and to disseminate across multiple continents over the past two decades. An *H*27 EAEC ST131 strain that has also acquired the $bla_{\text{CTX-M-101}}$ gene was causing bacteremia outbreaks in several geographic regions in Denmark, seemingly associated with recurrent infections. These findings emphasize the potential for different pathogens to evolve, thus potentially generating important new pathotypes that require continuous vigilance. They also illustrate the power of whole-genome sequencing to elucidate the historical and current molecular epidemiology and evolution of emerging pathogens of high public health importance.

## Materials and methods

### *Genome data and sequencing*

WGS was performed on a previously described international collection of 128 ST131 *E. coli* human isolates of serotype O25:H4 or O25:H- (1968 to 2011) from the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* (www.ssi.dk) (*10*). DNA samples were prepared for multiplexed, paired-end sequencing using a combination of Illumina MiSeq and HiSeq (*6, 10*). We also included a published *E. coli* ST131 sequence dataset comprising 93 US and German isolates of human and animal origin (1967 to 2011) (*6*), and sequences from 27 ESBL-producing ST131 bloodstream isolates (carrying $bla_{\text{CTX-M-101}}$) collected from Danish patients within the national surveillance program for antimicrobial resistance (DANMAP) for ESBL-producing *E. coli* (2014 – 2015) (*22*). Furthermore, all >3,500 *E. coli* ST131 genome data available at EnteroBase (http://enterobase.warwick.ac.uk, accessed November 15$^{th}$ 2017) were analyzed for EAEC characteristics (presence for the *aggR* gene) and positive isolates were included. Finally, we sequenced and included 13 *E. coli* isolates obtained from urine from the source patients for eight of the ESBL-producing blood isolates. The sequences were analyzed using the Bacterial Analysis Platform (BAP) from Center for Genomic Epidemiology (*44*).

*Plasmid sequencing and analysis*

Plasmid DNA was sequenced on both a MiSeq instrument (Illumina) and a MinION flow cell (Oxford Nanopore Technologies). The MiSeq library was made using the Nextera XT kit (Illumina) and sequencing was performed as a paired-end 250 bp run, yielding 372,720 reads with an average length of 237 bp. The MinION library was prepared using the Genomic Sequencing SQK-MAP006 kit and was sequenced on a FLO-MAP003 Early Access flow cell according to the manufacturer's instruction. Fast5 read files were subjected to base calling via a two-direction workflow using Metrichor software (ONT), yielding 4,856 passed 2D read files. Mixed assembly was performed by combining MiSeq and MinION reads using the SPAdes assembler (v3.9.0). Finally, CLCbio Genomics Workbench (v9.5.2) was used for end trimming of the assembled plasmid and for final error correction by mapping trimmed MiSeq reads against the plasmid contig obtained after the mixed SPAdes assembly.

pAA-ST131 was annotated using RAST (*45*), with putative hypothetical genes curated manually using NCBI BLASTn and BLASTp searches. A BLASTn atlas of pAA-ST131 and other pAA virulence plasmids was constructed using BLAST Ring Image Generator v0.95 (BRIG) (*46*).

*Virulence genotyping*

Isolates qualified molecularly as EAEC if positive for ≥1 of EAEC-associated putative virulence genes *aggR*, *aatA*, and *aaiC (10).* Isolates were regarded as ExPEC if positive for ≥2 of *papA* and/or *papC* (P fimbriae), *sfa* and *foc* (S and F1C fimbriae), *afa* and *dra* (Dr-binding adhesins), *kspM* II (group 2 capsule), and *iutA* (aerobactin siderophore system) (*24*). Isolates were considered UPEC if positive for ≥2 of *chuA* (heme uptake), *fyuA* (yersiniabactin siderophore system), *vat* (vacuolating toxin), and *yfcV* (adhesin) (*25*).

*Identification of SNPs*

SNPs in the core chromosome or plasmid, depending on analysis, were identified using the Northern Arizona SNP Pipeline (NASP) (*47*). Briefly, duplicate regions of the reference chromosome JJ1886 or pAA-ST131 plasmid (GenBank accession no. CP006784 and KY706108, respectively), were identified by aligning the reference against itself with NUCmer (*48*), followed by mapping of Illumina raw reads against the reference using the Burrows–Wheeler Aligner (BWA) (*49*) with identification of SNPs using GATK (*50*). Purging of recombinant region in the chromosome-based SNPs was performed using Gubbins v.2.2 (*51*).

*Phylogenetic analysis*

Relatedness between isolates according to core genome SNPs was inferred using RAxML v8.2.10 using the GTRCAT model (*52*). Relatedness of the plasmids was inferred using using PHyML with Smart Model Selection (*53*) with tree searching using SPR and 100 bootstrap replicates.

*Plasmid profiling*

Plasmids were purified as described by Kado and Liu (*54*) and visualized by separation on a 0.8% agarose gel electrophoresis and stained with GelRed (Biotium, Hayward, Ca, USA). *E. coli* strain 39R861, which contains four plasmids of sizes 147, 63, 36, and 7 kb (*55, 56*), served as a size marker.

*Statistical Analysis*

Comparisons of categorical variables were tested using Pearson's $\chi^2$ test with Yates' continuity correction, using R v3.3.2 statistical software (https://www.r-project.org). The criterion for statistical significance was $p<0.05$.

*Accession of Sequence data*

The accession numbers for the Illumina sequences generated from the 134 *E. coli* ST131 isolates presented in this study are available in the European Nucleotide Archive (ENA; https://www.ebi.ac.uk/ena) under the following accession numbers: PRJEB27194. Sequences can also be located in the ENA using the following study summary: "Emergence of enteroaggregative *Escherichia coli* within the ST131 lineage as a cause of extraintestinal infections". The sequence of the pAA-ST131 plasmid has been deposited in GenBank under accession number KY706108.

## Acknowledgements

We thank Michala T. Sørensen at Statens Serum Institut for performing the plasmid gel electrophoresis. We also thank Karin Sixhøj Pedersen at Statens Serum Institut for her excellent WGS work.

## Funding

This work was supported financially by the Danish Council for Research, grant DFF-1331-00161 to C. Struve, and the Danish Ministry of Health, as part of The Integrated Surveillance of ESBL/AmpC-producing *E. coli* and Carbapenemase-Producing Bacteria. This work was also supported in part by Office of Research and Development, (United States) Department of Veterans Affairs, grant #2I01CX000920-04 to J. Johnson.

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627
628

629 **Figure legends**

630 **Fig. 1.** Circular map of plasmid pAA-ST131 compared to other publicly available pAA plasmids.
631 The outer ring shows predicted ORFs of pAA-ST131. Colors represent different putative functions:
632 gray, hypothetical proteins; red, EAEC-specific virulence factors; blue, plasmid replication and
633 maintenance; maroon, catabolism and metabolism; orange, membrane and transporter proteins;
634 green, conjugational transfer proteins (*tra* and *trb* genes); light blue, regulatory genes; purple,
635 miscellaneous; and black, mobile elements. Within the circles representing pAA plasmids from other
636 EAEC strains (labeled one to five), the darkest color indicates >90% nucleotide identity, the lightest
637 color >80% identity.

638

639 **Fig. 2.** A) Unrooted phylogenetic tree of ST131 genomes from the WHO collection, the US/German
640 collection from 2010-2012, the Danish $bla_{\text{CTX-M-101}}$-containing isolates from 2014-2015, and the
641 EnteroBase database of international isolates carrying *fimH*27 (n=287). The distant *H*41 cluster is not
642 shown. EAEC isolates highlighted in red. B) Rooted phylogenetic tree with all isolates within the
643 *H*27 clade (n=79). ESBL-enzymes: CARB-2 (grey), CTX-M-101 (purple), CTX-M15 (pink), CTX-
644 M-15+OXA-10 (dark blue), CTX-M-27 (yellow) and SHV-12 (light blue). EAEC-positive isolates
645 are marked by an asterisk. Scale bar represents substitution rate in the conserved core genome.

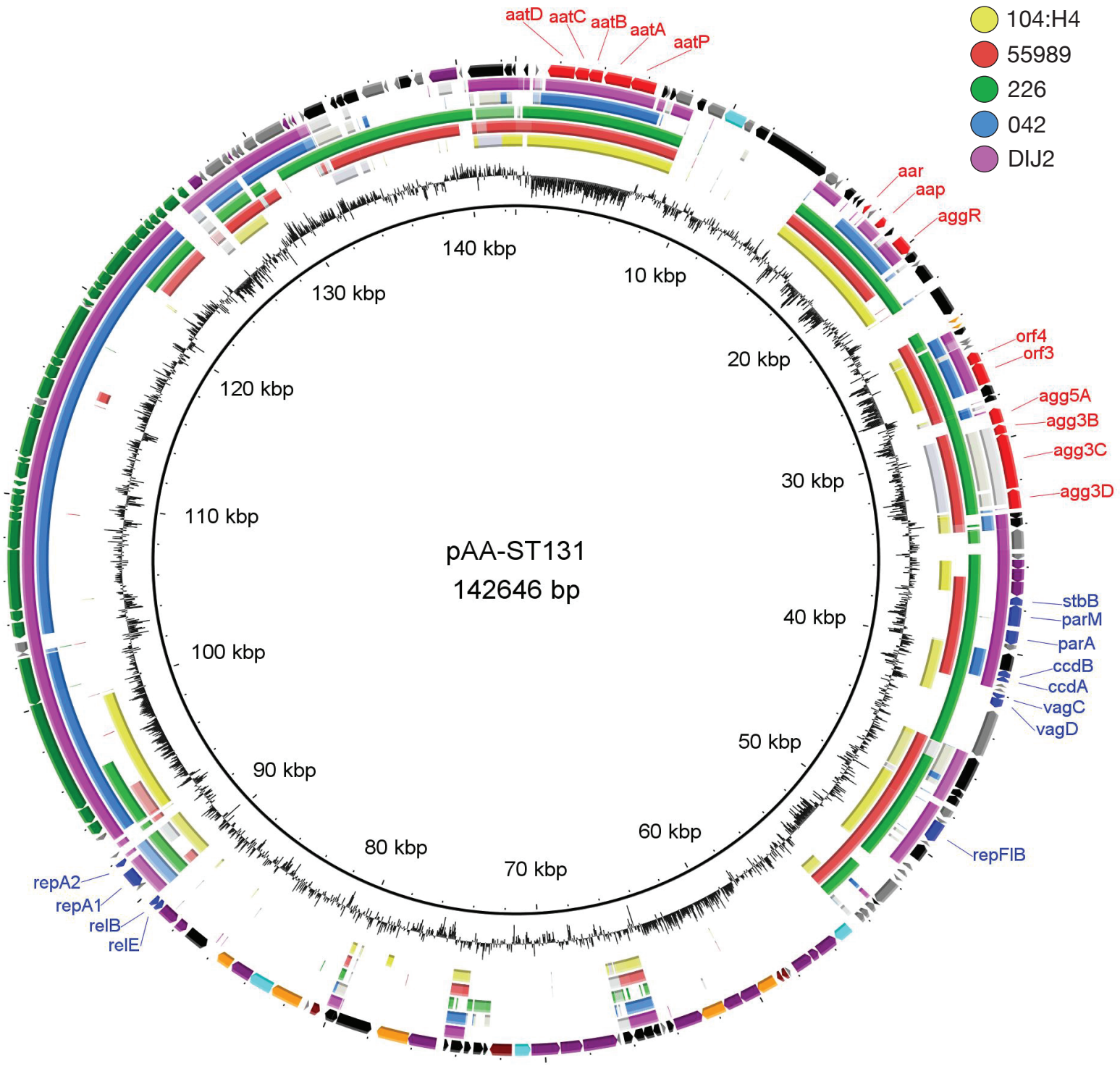
646

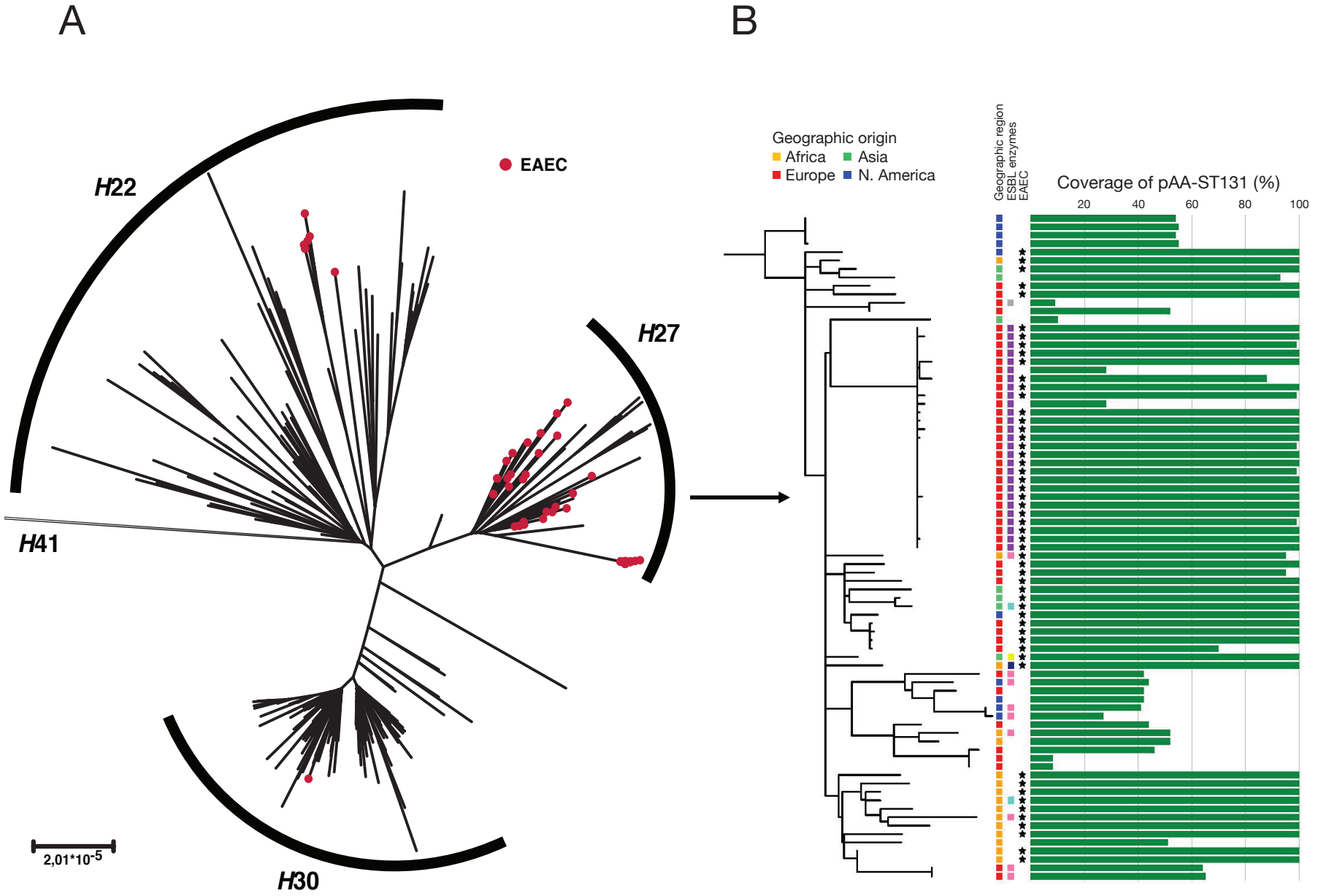
647 **Suppl. Fig. 1.** Rooted phylogenetic tree of ST131 *E. coli* strains from the WHO collection (n=121)
648 and the US/German collection (n=93). Isolates fulfilling the molecular criteria for EAEC are marked
649 with an asterisk.

650

651 **Suppl. Fig. 2.** Midpoint-rooted phylogeny based on 58 SNPs of paired blood (red) and urine
652 (yellow) isolates from eight Danish cases as well as all remaining Danish ST131 EAEC *H*27 $bla_{\text{CTX-M-101}}$-positive isolates. The analysis depicts the distinct relatedness of five corresponding blood and

654 urine isolates (Cases 1, 4, 5, 7, and 8), whereas the data is inconclusive for the remaining isolates
655 (Cases 2, 3 and 6).
656
657 **Suppl. Fig. 3**. Plasmid profiles of representative *fimH*27-carrying ST131 isolates either containing
658 $bla_{\text{CTX-M-101}}$ or from the WHO collection. Plasmid profiles with *E. coli* 39R861 as a marker (147, 63,
659 36 and 7kb) are included in lane 1. All ten *fimH*27 strains harbored only a single a plasmid. In seven
660 of the strains, this plasmid was 140-145 kb in size, whereas a slightly smaller size plasmid was
661 present in WHO isolate C1883-99. ESBL20150196 and ESBL20150300 both harbored a
662 significantly smaller plasmid of approximately 30 kb.
663
664 **Suppl. Fig. 4.** Phylogenetic tree of pAA from ST131 *H*27 isolates with more than 70% coverage of
665 pAA-ST131 from representative Danish isolate ESBL20150001. Three of the *H*22/*H*298 EAEC
666 isolates from the WHO collection (C156-00, C168-00 and C180-00) were included as an outgroup.
667 A total of 197 SNPs was identified equivalent to approximately 25% of pAA-ST131.




A
B
EAEC
H22
H27
H41
H30
2,01*10-5
Geographic origin
Africa
Asia
Europe
N. America
Geographic region
ESBL enzymes
EAEC
Coverage of pAA-ST131 (%)
20
40
60
80
100