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Endophytic Lifestyle of Global Clones of Extended-Spectrum β-Lactamase-Producing Priority Pathogens in Fresh Vegetables: a Trojan Horse Strategy Favoring Human Colonization?

Ralf Lopes,[a] Danny Fuentes-Castillo,[b,c] Herrison Fontana,[c,d] Larissa Rodrigues,[c,d] Karine Dantas,[a] Louise Cerdeira,[e] Isabel Henriques,[f,g] Nilton Lincopan[a,c,d]

[a]Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil
[b]Department of Pathology, School of Veterinary Medicine and Animal Sciences, University of São Paulo, São Paulo, Brazil
[c]One Health Brazilian Resistance Project (OneBR), São Paulo, Brazil
[d]Department of Clinical Analysis, School of Pharmacy, University of São Paulo, São Paulo, Brazil
[e]Department of Infectious Diseases, Central Clinical School, Monash University, Melbourne, Australia
[f]Centre for Environmental and Marine Studies (CESAM), University of Aveiro, Aveiro, Portugal
[g]Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra, Coimbra, Portugal

ABSTRACT The global spread of antibiotic-resistant bacteria and their resistance genes is a critical issue that is no longer restricted to hospital settings, but also represents a growing problem involving environmental and food safety. In this study, we have performed a microbiological and genomic investigation of critical priority pathogens resistant to broad-spectrum cephalosporins and showing endophytic lifestyles in fresh vegetables sold in a country with high endemicity of extended-spectrum β-lactamases (ESBLs). We report the isolation of international high-risk clones of CTX-M-15-producing *Escherichia coli*, belonging to clonal complexes CC38 and CC648, and *Klebsiella pneumoniae* of complex CC307 from macerated tissue of surface-sterilized leaves of spinach, cabbage, arugula, and lettuce. Regardless of species, all ESBL-positive isolates were able to endophytically colonize common bean (*Phaseolus vulgaris*) seedlings, showed resistance to acid pH, and had a multidrug-resistant (MDR) profile to clinically relevant antibiotics (i.e., broad-spectrum cephalosporins, aminoglycosides, and fluoroquinolones). Genomic analysis of CTX-M-producing endophytic *Enterobacterales* revealed a wide resistome (antibiotics, biocides, disinfectants, and pesticides) and virulome, and genes for endophytic fitness and for withstanding acidic conditions. Transferable IncFIB and IncHI2A plasmids carried $bla_{CTX\text{-}M\text{-}15}$ genes and, additionally, an IncFIB plasmid (named pKP301cro) also harbored genes encoding resistance to heavy metals. These data support the hypothesis that fresh vegetables marketed for consumption can act as a figurative Trojan horse for the hidden spread of international clones of critical WHO priority pathogens producing ESBLs, and/or their resistance genes, to humans and other animals, which is a critical issue within a food safety and broader public and environmental health perspective.

IMPORTANCE Extended-spectrum β-lactamases (ESBL)-producing *Enterobacterales* are a leading cause of human and animal infections, being classified as critical priority pathogens by the World Health Organization. Epidemiological studies have shown that spread of ESBL-producing bacteria is not a problem restricted to hospitals, but also represents a growing problem involving environmental and food safety. In this regard, CTX-M-type β-lactamases have become the most widely distributed and clinically relevant ESBLs worldwide. Here, we have investigated the occurrence and genomic features of ESBL-producing *Enterobacterales* in surface-sterilized fresh vegetables. We have uncovered that international high-risk clones of CTX-M-15-producing *Escherichia coli* and *Klebsiella pneumoniae* harboring a wide resistome and virulome,

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Address correspondence to Nilton Lincopan, lincopan@usp.br.

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carry additional genes for endophytic fitness and resistance to acidic conditions. Furthermore, we have demonstrated that these CTX-M-15-positive isolates are able to endophytically colonize plant tissues. Therefore, we believe that fresh vegetables can act as a figurative Trojan horse for the hidden spread of critical priority pathogens exhibiting endophytic lifestyles.

KEYWORDS *E. coli* ST648, *E. coli* ST38, *K. pneumoniae* CC307, CTX-M-15, food, One Health, ESBL

Extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria are a leading cause of human and animal infection, being categorized as critical priority pathogens by the World Health Organization (1). In this regard, plasmid-mediated ESBLs of the CTX-M family have been widely identified in different genera of *Enterobacterales*, with CTX-M-15 being the most clinically relevant ESBL worldwide (2). Curiously, *Kluyvera* species, bacteria commonly found in the rhizosphere and endophytic ecosystems, have been proposed as the original source of $bla_{\text{CTX-M}}$-type genes (3). Therefore, endophytic bacteria that colonize internal tissues of vegetables can represent a hidden mode of transmission of virulent and/or antibiotic-resistant bacteria and their resistance genes to humans and other animals (4–7).

Currently, epidemiological studies have shown that the spread of CTX-M-producing bacteria is not a problem restricted to hospitals, but also represents a growing problem involving environmental and food safety (2). On the other hand, rates of CTX-M-producing *Enterobacterales* in community-acquired urinary tract infections (UTIs) and community fecal carriage have increased significantly worldwide, with developing countries being the most affected (8, 9). In this regard, various factors, such as environmental sources, international travel, and wild, companion, and food-producing animals, have accelerated the global spread of CTX-M ESBLs in the community, mainly in countries with endemic status (8, 10–12).

Specifically, contamination of fresh vegetables by critical priority pathogens is the greatest concern (6, 7, 13, 14), since these foods are consumed raw and this increases the risk of human exposure to ESBL producers and other antibiotic-resistant bacteria with clinical interest (15). Although ingestion of ESBL-producing bacteria may not have an immediate clinical health implication, colonization by this pathway can contribute to the transfer of antibiotic resistance genes to other bacterial species present in the gut microbiota (7, 14, 16). Consequently, a potential threat to human health would be associated with future endogenous infections, mainly in immunosuppressed patients, where therapeutic failure could occur.

Even though clinically significant ESBL-producing *Enterobacterales*, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*, have been frequently found as epiphytes on fresh vegetables (13, 14), little is known about their endophytic existence. Therefore, we have performed a microbiological and genomic investigation of critical priority pathogens displaying resistance to broad-spectrum cephalosporins and showing endophytic lifestyles in fresh vegetables sold in a country with high endemicity of ESBLs.

RESULTS

Multidrug-resistant ESBL-producing endophytic *Enterobacterales* isolated from fresh vegetables. The presence of endophytic ESBL-producing *Enterobacterales* was confirmed in 10.4% of 48 fresh vegetables samples screened after surface sterilization, including spinach (2 positive samples for ESBL-producing *E. coli* strain ESP110 and *E. cloacae* strain ESP151), cabbage (1 positive sample for 2 ESBL-producing *E. coli* strains [REP215 and REP237]), lettuce (1 positive sample for ESBL-producing *K. pneumoniae* [strain ALF301]), and arugula (1 positive sample for ESBL-producing *K. pneumoniae* [strain RUC232]). All strains displayed a multidrug-resistant profile (17) with high MIC values above resistance breakpoints for broad-spectrum cephalosporins (Table 1). Further resistance to fluoroquinolones was detected in *K. pneumoniae* RUC232 and *E.*

TABLE 1 Antimicrobial resistance profile of endophytic ESBL (CTX-M-15)-producing *Enterobacterales* from commercial vegetables

			MIC (μg/ml)[b]																		
Strain	Vegetable	Geographical coordinates[a]	Ptz	Cef	Cro	Caz	Fep	Fox	Atm	Ipm	Mem	Etp	Gen	Ami	Nal	Cip	Enr	Lvx	Mxf	Sxt	Chl	Tet
E. cloacae ESP151	Spinach	23°23′24.0″ S; 47°07′48.0″ W	**≥256**	**≥256**	**≥32**	8	12	**128**	**32**	0.75	0.094	0.25	**16**	4	16	0.75	0.09	0.5	0.06	**≥32**	**≥256**	6
K. pneumoniae ALF301	Lettuce	23°32'03.8″ S; 46°44'08.4″ W	4	**≥256**	**≥32**	8	**24**	4	**48**	0.25	0.023	0.09	**≥1024**	4	3	0.12	0.04	0.06	0.03	**≥32**	**≥256**	**64**
K. pneumoniae RUC232	Arugula	23°13′48.0″ S; 46°11'24.0″ W	**≥256**	**≥256**	**≥32**	**16**	12	4	**16**	0.38	0.032	0.25	1	8	**≥32**	**16**	**4**	0.5	0.06	2	6	**16**
E. coli ESP110	Spinach	23°23′24.0″ S; 47°07'48.0″ W	**≥256**	**≥256**	**≥32**	**16**	4	12	**32**	0.25	0.094	0.19	**16**	8	16	1	0.04	0.25	0.03	**≥32**	**≥256**	**64**
E. coli REP215	Cabbage	23°25′12.0″ S; 47°15′00.0″ W	4	**≥256**	**≥32**	8	**16**	12	**16**	0.19	0.047	0.25	**48**	16	**≥256**	**≥32**	**≥32**	**≥32**	**≥32**	**≥32**	6	1.5
E. coli REP237	Cabbage	23°25′12.0″ S; 47°15′00.0″ W	**≥256**	**≥256**	**≥32**	12	**≥32**	3	**24**	0.38	0.032	0.19	0.5	4	6	0.5	0.06	0.25	0.12	**≥32**	3	**192**

[a]Geographical coordinates indicate origin of cultivated vegetables.

[b]Ptz, piperacillin-tazobactam; Cef, cephalothin; Cro, ceftriaxone; Caz, ceftazidime; Fep, cefepime; Fox, cefoxitin; Atm, aztreonam; Ipm, imipenem; Mem, meropenem; Etp, ertapenem; Gen, gentamicin; Ami, amikacin; Nal, nalidixic acid; Cip, ciprofloxacin; Enr, enrofloxacin; Lvx, levofloxacin, Mxf, moxifloxacin; Sxt, sulfamethoxazole/trimethoprim; Chl, chloramphenicol; Tet, tetracycline. Resistant MIC values are shown in boldface type, with resistance profiles determined using the CLSI 2020 guidelines (69). For enrofloxacin and moxifloxacin, resistance profiles were determined using CLSI 2018 (70) and EUCAST 2021 (https://www.eucast.org/) guidelines, respectively.

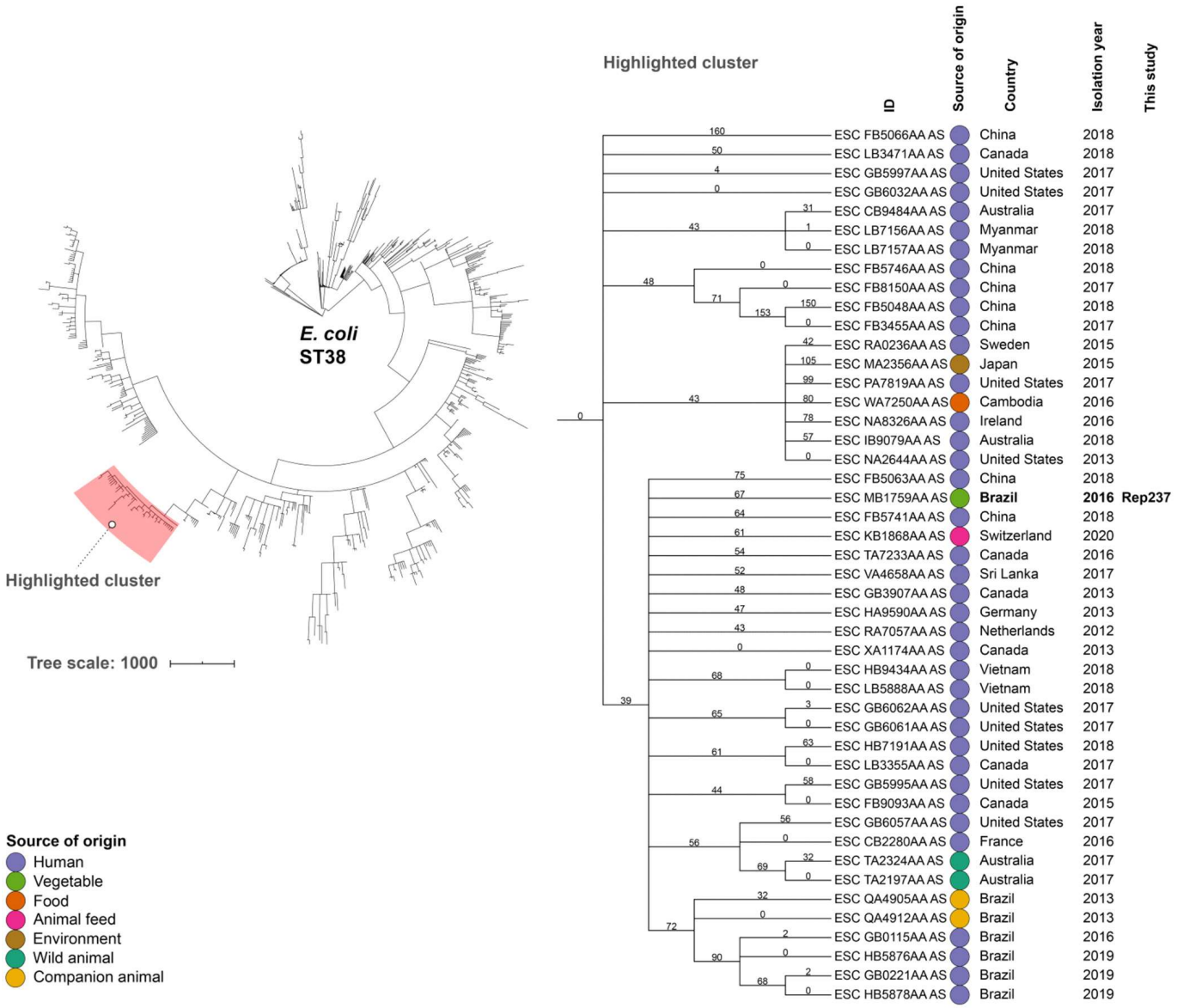

FIG 1 Phylogenomic analysis of a CTX-M-15-positive *Escherichia coli* REP237 strain, isolated from cabbage, in relation to an international collection of genomes of *E. coli* strains belonging to sequence type (ST) 38. On the left, the image shows a minimum spanning tree based on wgMLST of 799 worldwide distributed *E. coli* strains belonging to ST38, constructed by the MSTree V2 tool from EnteroBase. The *E. coli* strain REP237 was organized in the cluster highlighted in red. The highlighted cluster includes a partial depiction of the tree, including the Enterobase identification (ID), source of origin, country, and isolation year of genomically related isolates. The figure was generated with iTOL v.5.5 (https://itol.embl.de). An interactive version of the tree can be found at https://itol.embl.de/tree/14310712557248381595353218.

coli REP215 strains, whereas all endophytic ESBL-positive strains remained susceptible to carbapenems (i.e., imipenem, meropenem, and ertapenem) and amikacin (Table 1).

Identification of global clones and phylogenomic analysis. Endophytic ESBL-producing isolates belonged to different sequence types (ST). In this regard, *E. coli* ESP110, REP215, and REP237 from spinach and cabbage belonged to ST4012 and the international ST648 and ST38, respectively. *K. pneumoniae* ALF301 belonged to ST198 and *K. pneumoniae* RUC232 belonged to the new sequence type ST2739, a single-locus variant of international ST307. *E. cloacae* ESP151 isolated from spinach was assigned to the new ST927.

Genomic relatedness analysis of 798 assembled genomes of globally reported *E. coli* ST38 assigned *E. coli* strain REP237 to a cluster comprising human *E. coli* genomes from Asia, Europe, and America (including Brazilian human isolates), two wild animal *E. coli* genomes from Australia, one animal feed *E. coli* genome from Switzerland, and two companion animal genomes from Brazil (Fig. 1). On the other hand, the minimum

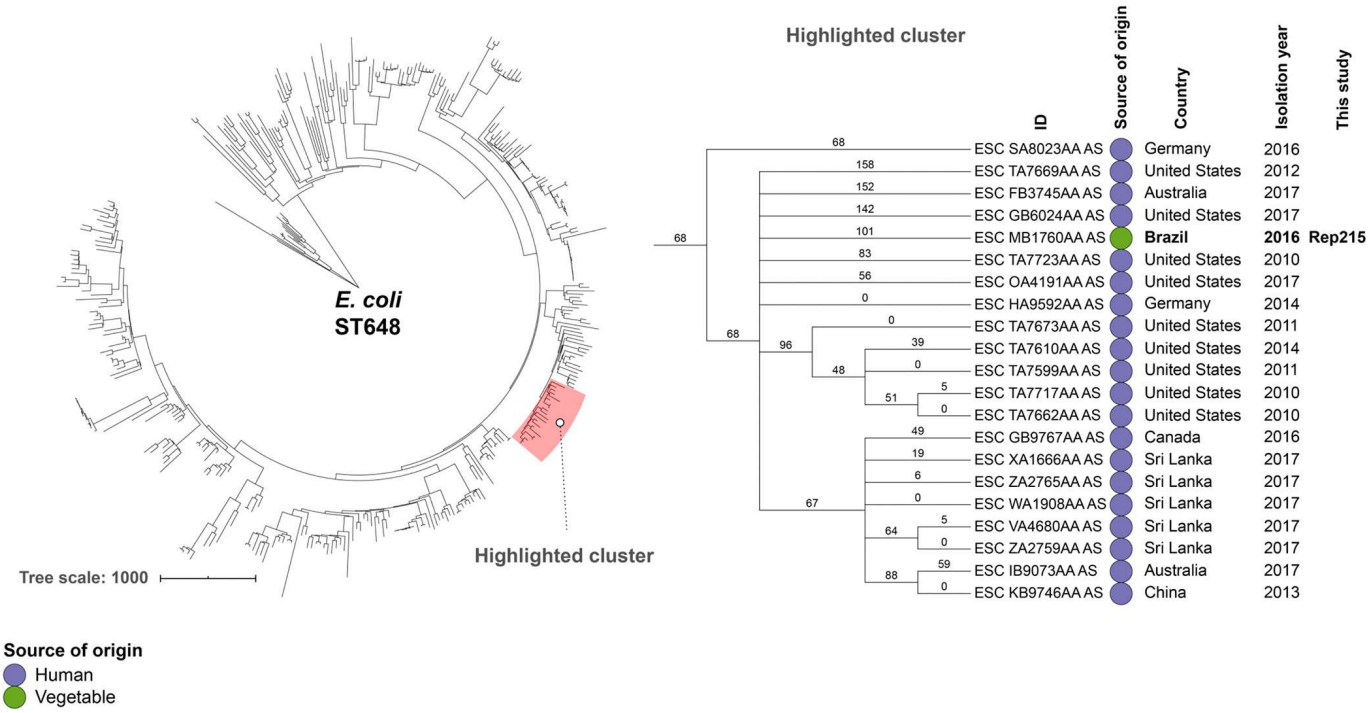

FIG 2 Phylogeny of a CTX-M-15-producing *E. coli* REP215 strain, isolated from cabbage, in relation to an international collection of genomes of *E. coli* strain belonging to ST648. On the left, the image shows a minimum spanning tree based on wgMLST of 390 worldwide distributed *E. coli* strains belonging to ST648, constructed by the MSTree V2 tool from EnteroBase. The *E. coli* strain REP215 was organized in the cluster highlighted in red. The highlighted cluster includes a partial depiction of the tree, including the Enterobase identification (ID), source of origin, country, and isolation year of genomically related isolates. The figure was generated with iTOL v.5.5 (https://itol.embl.de). An interactive version of the tree can be found at https://itol.embl.de/tree/14310712557248341595353217.

spanning tree for *E. coli* REP215 isolate and the other 389 genome assemblies belonging to ST648 assigned *E. coli* REP215 to a cluster comprising human genomes from Europe, Asia, America, and Oceania (Fig. 2).

While genomes of *E. cloacae* and *K. pneumoniae* strains belonging to ST927 and ST2739, respectively, were not publicly available for comparative phylogenetic analysis, comparative core-genome multilocus sequence type (cgMLST) analysis of endophytic *K. pneumoniae* strain ALF301 with human *K. pneumoniae* strains belonging to ST198, previously identified in Brazil, revealed that endophytic *K. pneumoniae* ALF301 differs in 23 and 72 cgMLST alleles from human *K. pneumoniae* ICBKpBL-III-03(1) (GenBank acession number NIHK00000000.1) and ICBKpBL-III-02(1) (GenBank acession number: NGJM00000000.1) strains, respectively.

Resistome, virulome, and identification of endophytic and acid tolerance genes. Whole-genome sequence (WGS) analysis revealed that in all endophytic strains, ESBL production was associated with the presence of $bla_{\text{CTX-M-15}}$ genes (Fig. 3). Additionally, the $bla_{\text{OXA-1}}$ β-lactamase gene was further identified in all strains, except in *E. coli* ESP110. On the other hand, while *E. coli* strains ESP110 and REP215, *K. pneumoniae* RUC232, and *E. cloacae* ESP151 carried the $bla_{\text{TEM-1B}}$ β-lactamase gene, both *K. pneumoniae* strains were also bla_{SHV}-positive. In addition to beta-lactam resistance genes, the presence of resistance determinants to aminoglycosides (*strA*, *strB*, *aac(3)−II*, *aac(6′)Ib−cr*, *aadA5* and *ant(3″)Ia*), quinolones (*aac(6′)Ib−cr*, *qnrB1*, *oqxA*, and *oqxB*), sulfonamides (*sul1* and *sul2*), trimethoprim (*drfA14* and *drfA17*), phenicols (*catA1*, *cmlA1*, and *floR*), tetracyclines (*tetA* and *tetB*), fosfomycin (*fosA*) and macrolides (*ermB* and *mphA*) was confirmed among endophytic strains (Fig. 3). Substitutions Thr-83→Ile and Ser-80→Ile in the quinolone resistance-determining region (QRDR) of GyrA and ParC, respectively, were identified in the quinolone-resistant *K. pneumoniae* RUC232 strain, whereas substitutions Ser-83→Leu and Asp87→Asn in GyrA, and Ser-80→ Ile in ParC were found in *E. coli* REP215 (Fig. 3).

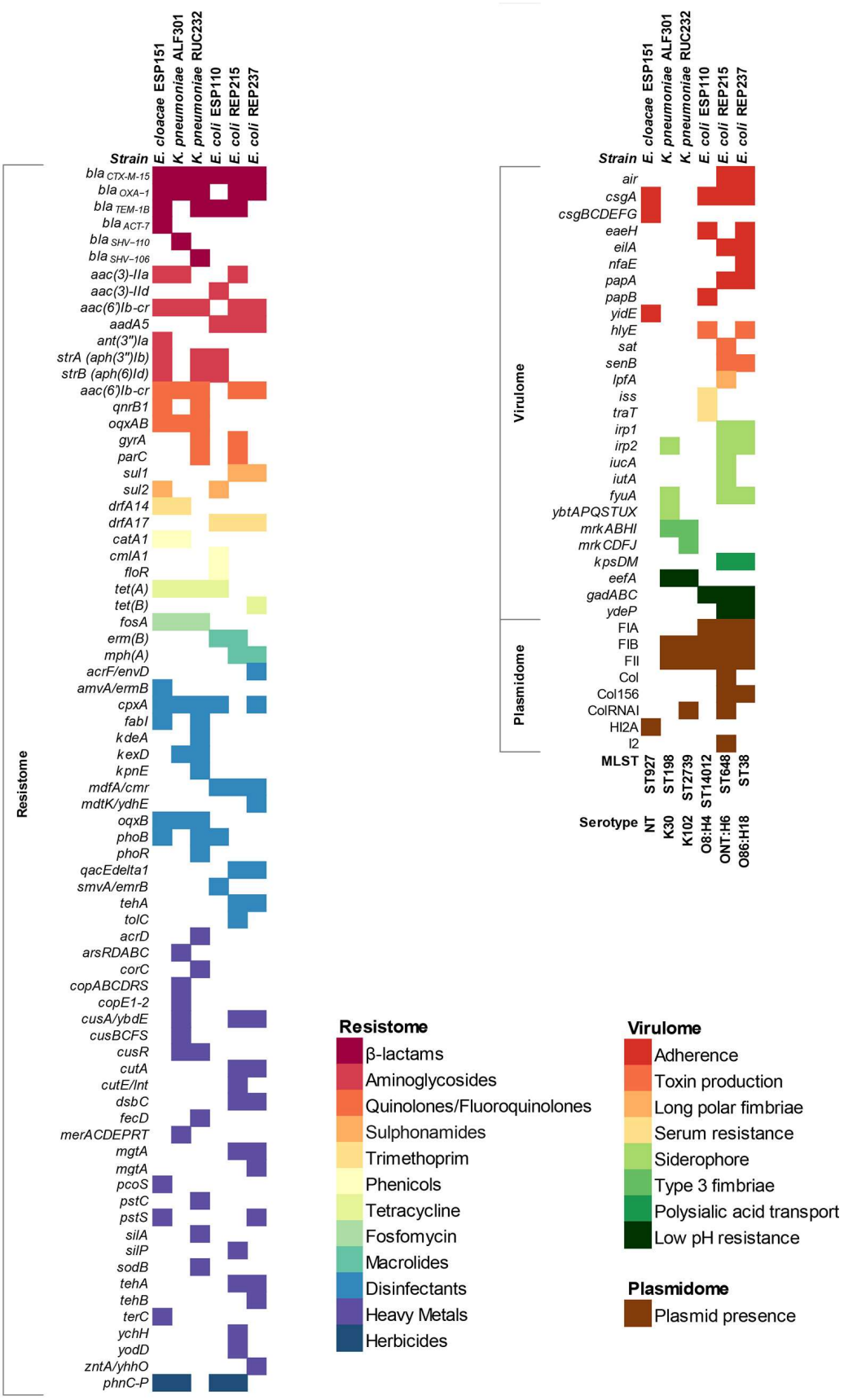

FIG 3 Heat map of resistome, virulome, plasmidome, and MLST and serotype data generated from whole-genome comparative genomics for endophytic multidrug-resistant *Enterobacterales* isolated from commercial vegetables. NT, not typed. For all predicted genes, a >90% identity threshold was used as a filter for identification.

Heavy metals resistance gene clusters, such as *cusSRCFBA*, *copE2ABCDRSE1*, *arsRDABC*, and *merRTPCAD*, encoding resistance to copper/silver, copper, arsenic, and mercury, respectively, were identified in *K. pneumoniae* ALF301 isolated from commercial lettuce (Fig. 3), whereas *E. coli* REP237 carried tellurite resistance genes *tehA/tehB*. On the other hand, *E. coli* REP215, *E. coli* ESP110, *K. pneumoniae* ALF301, and *E. cloacae* ESP151 harbored the *phnC-P* gene system conferring resistance to glyphosate herbicide.

Regarding antiseptics and disinfectants, genes predicted to confer tolerance to hydrogen peroxide (*cpxA* and *kpnE*) and resistance to quaternary ammonium compounds (*acrF/envD*, *amvA/ermB*, *cpxA*, *kpnE*, *mdfA/cmr*, *mdtK/ydhE*, *oqxB*, *phoB*, *phoR*, *qacEdelta1*, *smvA/emrB*, *tehA*, and *tolC*), phenol (*tolC*), triclosan (*fabI*, *kpnE*, *oqxB*, and *tolC*), biguanides/chlorhexidine (*cpxA*, *kpnE*, *oqxB*, *phoB*, *phoR*, and *qacEdelta1*), organosulfate/sodium dodecyl sulfate compounds (*acrF/envD*, *amvA/ermB*, *kdeA*, *kpnE*, *oqxB*, *qacEdelta1*, and *tolC*), and/or ionic detergents/sodium deoxycholate (*kexD*, *kpnE*, *mdtK/ydhE*, and *qacEdelta1*) were also identified in all CTX-M-15-positive endophytic isolates (Fig. 3).

Virulome analysis of *E. coli* strains revealed the presence of genes involved in adherence (*air*, *eilA*, and *nfaE*), toxin production (*sat* and *senB*), long polar fimbriae (*lpfA*), increased serum survival (*iss*), and acid resistance (*gad*). In this regard, *E. coli* REP215 and REP237 belonged to the phylogroup D known for including highly virulent lineages, whereas *E. coli* ESP110 was assigned to the low-virulence phylogroup A, common among commensal lineages (18). Virulome analysis of *K. pneumoniae* strains confirmed genes encoding the production (*irp2* and *ybt*) and uptake (*fyuA*) of the siderophore yersiniabactin, and/or genes encoding type 3 fimbriae (*mrk* gene cluster) (Fig. 3). On the other hand, *K. pneumoniae* RUC232 showed an identical capsular polysaccharide serotype (KL102-*wzi*173) and O-locus (O2v2) than *K. pneumoniae* strains belonging to clonal complex CC307 (19), whereas *K. pneumoniae* ALF301 of ST198 displayed *ybt*16, ICEKp12, *wzi*85, KL30, and O1v1 serotype. In *E. cloacae* ESP151, genes involved in hyperadherence (*yidE*) and curli fimbriae formation (*csgABCDEFG* operon) were predicted (Fig. 3).

Genetic determinants contributing to an endophytic lifestyle, such as genes for nitrogen supply favoring plant growth (*narI*, *narJ*, and *nirB*), were found in all CTX-M-15-positive strains (20, 21). On the other hand, genes for biosynthesis of 2,3-butanediol, involved in plant growth (22), were found in all *K. pneumoniae* and *E. cloacae* strains. Furthermore, genes encoding chitinase ChiC (EC 3.2.1.14) were identified in *E. cloacae* ESP151, *K. pneumoniae* ALF301 and RUC232, and *E. coli* ESP110 strains. Genes encoding cellulase A 3 (EC 3.2.1.4) were harbored by all endophytic ESBL producers.

Plasmidome, horizontal transfer of plasmids, and genetic environments of *bla*$_{\text{CTX-M-15}}$ ESBL genes. IncFIB and IncFII plasmid replicon types were harbored by CTX-M-15-producing endophytic strains. However, while in *E. coli* and *K. pneumoniae* strains the *bla*$_{\text{CTX-M-15}}$ gene was carried on IncFIB plasmids, in *E. cloacae* this gene was harbored by an IncHI2A plasmid (Fig. 3). Conjugation assays confirmed transfer of *bla*$_{\text{CTX-M-15}}$/IncFIB plasmids from *E. coli* strains REP215 (ST648), REP237 (ST38), and ESP110 (ST4012) at frequencies of 8.67×10^{-4}, 2.33×10^{-3}, and 5.14×10^{-3} transconjugants/recipient cell, respectively. For *K. pneumoniae* RUC232 and ALF301, and *E. cloacae* ESP151, transfer of plasmid carrying the *bla*$_{\text{CTX-M-15}}$ gene was only achieved by transformation with efficiency of 4.95×10^5, 6.12×10^5, and 1.05×10^6 transformants/μg of plasmid, respectively.

In *K. pneumoniae* ALF301, the *bla*$_{\text{CTX-M-15}}$ gene was carried on an IncFIB plasmid named pKP301cro. The pKP301cro plasmid is 147,442 bp in size, with G+C content of 50.78%, coharboring *cusSRCFBA*, *copE2ABCDRSE1*, and *arsRDABC* gene clusters (Fig. 4A and C). Interestingly, this plasmid showed significant divergence from others of the same incompatibility group identified in clinical and environmental strains (Fig. 4B).

Three different genetic environments were found surrounding *bla*$_{\text{CTX-M-15}}$ (Fig. 5). In *K. pneumoniae* and *E. cloacae* strains and one *E. coli* strain (REP215), the international *bla*$_{\text{CTX-M-15}}$ genetic environment was confirmed (Fig. 5A) (23). Moreover, two novel environments were present in the endophytic *E. coli* strains belonging to ST38 (REP237)

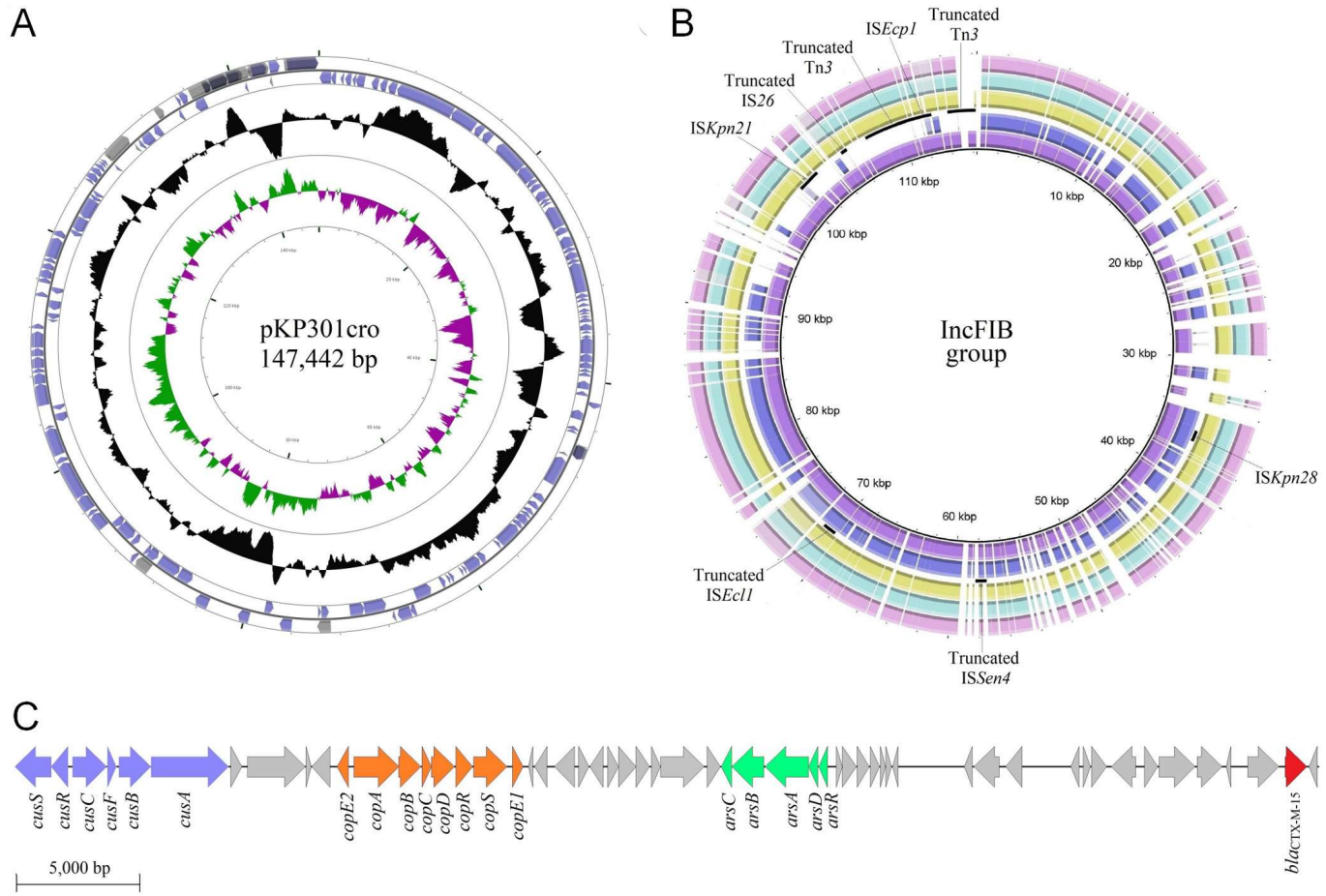

FIG 4 (A) Overview of the pKP301cro plasmid harbored by the endophytic *K. pneumoniae* ALF301 ST198 isolated from lettuce. Blue, protein coding sequences; gray, transposable elements; black, GC content; green, positive GC skew; purple, negative GC skew. (B) Comparison of IncFIB plasmids carrying β-lactamase genes. Low identities (70% to 50%) between plasmid proteins are indicated as lighter shades. Matches with less than 50% identity and no matches appear as blank spaces. In pink, pLGP4 plasmid (GenBank: MF116002.1) from uncultured bacterium; in green, p6234-198.371kb plasmid (GenBank: CP010390.1); in yellow, pKPSH11 plasmid (GenBank: KT896504.1); in blue, pKP301cro plasmid (GenBank: KY495890.1); and in purple, pKPN3-307_typeD plasmid (GenBank: KY271407.1) of *K. pneumoniae* strains from bodily fluid, wastewater, lettuce, and aspirate bronchial, respectively. Black arcs indicate transposable elements in pKP301cro. (C) Region from pKP301cro showing resistance genes to silver/copper (blue, *cusSRCFBA*), copper (orange, *copE2ABCDRSE1*), arsenic (green, *arsRDABC*), and β-lactams (red, $bla_{\text{CTX-M-15}}$).

and ST4012 (ESP110) isolated from cabbage and spinach, respectively (Fig. 5B and C). In these novel environments, the ST38 lineage showed an 1,171-bp IS*Ecp1* insertion element truncated by an incomplete IS*26* upstream of the $bla_{\text{CTX-M-15}}$ gene, while the ST4012 lineage exhibited a 494-bp IS*Ecp1* truncated by an inverted IS*26*.

Endophytic properties of CTX-M-15-producing *Enterobacterales*. This assay was designed to exclusively evaluate endophytic properties and plant-colonizing abilities of ESBL-producing isolates by using a common bean (*Phaseolus vulgaris*) model, determining endophytic bacterial loads recovered from root and shoot tissues (leaves) after inoculation of sterile sprouts obtained from surface-sterilized bean seeds (Table 2). In this regard, all CTX-M-15-positive isolates were able to endophytically colonize common bean seedlings. In order to evaluate endophytic properties and plant-colonizing abilities, bacterial burdens were evaluated at 15 days after inoculation of common bean. All strains efficiently colonized the interior of the root and shoot systems, supporting endophytic behaviors. Significantly higher bacterial counts in the root ($P < 0.05$) were determined for CTX-M-15-producing *K. pneumoniae* ALF301 and RUC232. Otherwise, the *E. cloacae* ESP151 displayed a higher bacterial burden within the shoot than the other strains ($P < 0.05$) (Table 2).

Tolerance of CTX-M-15-producing endophytic *Enterobacterales* to acid pH. Initially, all *Enterobacterales* strains were grown in Trypticase soy broth (TSB) medium

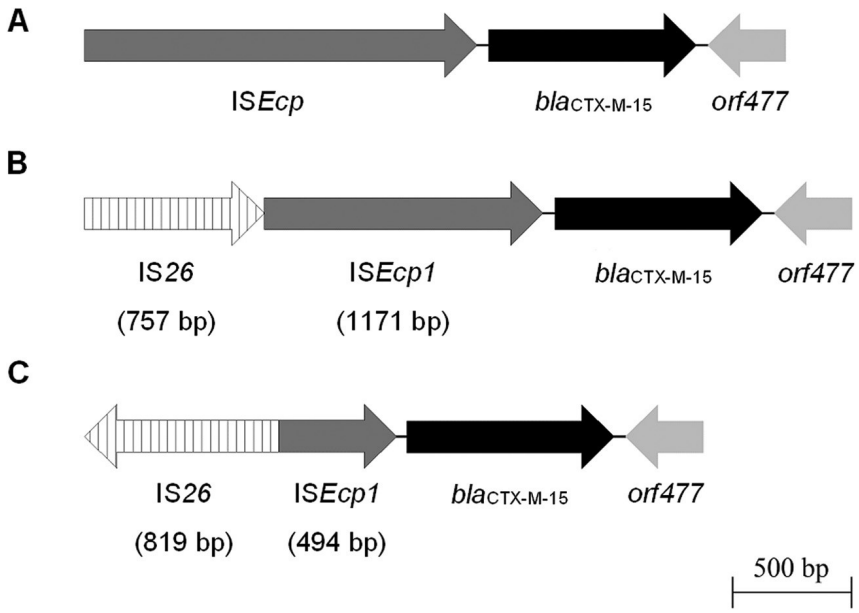

FIG 5 Schematic representation of genetic environments surrounding $bla_{CTX\text{-}M\text{-}15}$ in endophytic *Enterobacterales*. (A) International $bla_{CTX\text{-}M\text{-}15}$ genetic environment (23), identified in endophytic *E. coli* of ST648 (REP215, GenBank accession number: MG844172), *K. pneumoniae* of ST198 and ST2739 (ALF301, GenBank accession number: MG844168, and RUC232, GenBank accession number: MG844173, respectively), and *E. cloacae* (ESP151, GenBank accession number: MG844171) strains. (B) The novel environment identified in *E. coli* REP237 of ST38 (GenBank accession number: MG844170) presents a 1,171-bp IS*Ecp1* truncated by an incomplete IS*26* upstream of the $bla_{CTX\text{-}M\text{-}15}$ gene. (C) The novel environment identified in *E. coli* ESP110 of ST4012 (GenBank accession number: MG844169) presents a 494-bp IS*Ecp1* truncated by an inverted IS*26*.

at pH 7.0 to a cell density of $\sim 1 \times 10^8$ CFU/ml, and the cells were collected, washed, and transferred into the same medium at pH ranging from 7.0 to 2.0, at a final concentration of 10^5 cells per well. For all strains, no reduction of CFU/ml was observed after 24 h of incubation at pH 6.0 to 5.0. However, at pH 4.0, while no reduction in CFU/ml of *K. pneumoniae* was observed after 24 h of incubation, for *E. coli* and *E. cloacae* the cell density was reduced by 2 to 3 log CFU/ml. On the other hand, pH 3.0 led to reduction of *K. pneumoniae* and *E. coli* cell densities by 3 to 5 and 2 to 4 log CFU/ml at 1 h and 2 h of incubation, respectively, whereas for all strains CFU/ml were undetectable after 24 h of incuba-

TABLE 2 Endophytic bacterial load in root and shoot tissues of common bean (*Phaseolus vulgaris*) inoculated with CTX-M-15-producing *Enterobacterales* isolated from commercial vegetables

	CFU/g tissue ± SD	
Inoculating strain[d]	Root	Shoot
K. pneumoniae ALF301[a]	$1.6 \pm 0.1 \times 10^5$	$6.9 \pm 0.4 \times 10^2$
K. pneumoniae RUC232[a]	$8.7 \pm 0.5 \times 10^4$	$3.8 \pm 0.3 \times 10^2$
E. cloacae ESP151[b]	$6.4 \pm 0.4 \times 10^4$	$2.1 \pm 0.1 \times 10^3$
E. coli REP215	$3.9 \pm 0.4 \times 10^4$	$7.0 \pm 0.9 \times 10^1$
E. coli ESP110	$3.2 \pm 0.6 \times 10^4$	$3.1 \pm 0.5 \times 10^2$
E. coli REP237	$5.1 \pm 0.5 \times 10^3$	$7.5 \pm 1.0 \times 10^1$
A. baumannii ATCC 19606	-[c]	-[c]

[a]Significantly higher bacterial counts in the root ($P < 0.05$).
[b]Significantly higher bacterial counts in the shoot ($P < 0.05$).
[c]Undetectable bacterial colonies.
[d]Common beans (*Phaseolus vulgaris*) grains were surface-sterilized and, after a two-day germination, sprouts were incubated for 30 min with bacterial suspension (OD_{600} = 1.5) and transferred to plant culture bottles with Murashige and Skoog medium. Endophytic bacterial load in root and shoot tissue was assessed at 15 days after inoculation. All assays were performed in triplicate. *Acinetobacter baumannii* ATCC 19606 was used as a negative control for endophytic colonization.

tion. Finally, only *E. coli* strains presented tolerance to pH 2.0, where cell density was reduced by 3 to 4 and 2 to 3 log CFU/ml at 1 h and 2 h of incubation, respectively.

DISCUSSION

Members of the *Enterobacterales* order have been shown to colonize and benefit plant growth in various crops, such as wheat, maize, rice, and cucumber (4, 24–27). Worryingly, ESBL-producing *Enterobacterales*, including CTX-M-15 producers, have also been reported in vegetables, representing a risk of human exposure to MDR critical priority pathogens through this food source (7, 13, 14). In this regard, the occurrence of epiphytic ESBL producers in fresh vegetables has been described in North American, Asian, and European countries (13, 28). In South America, ESBL-positive *E. coli* of types ST44 and ST410 have been recently identified in fresh vegetables sold in Ecuador (14).

In Brazil, the largest and most populated country in South America, ESBL production has been documented to be more challenging than in developed countries. In fact, ESBL-producing *Enterobacterales* are endemic in both hospital and community settings (29–31). *K. pneumoniae* and *E. coli* have been frequently associated with the production of CTX-M-15 ESBLs (32). Worryingly, in this country, CTX-M-15 producers have also been identified in chicken meat, wild and food-producing animals, pets, Amazonian fish, and aquatic environment samples (11, 33–44), whereas its presence in vegetables has not been investigated in deep, so far.

Most studies conducted to evaluate contamination of commercial vegetables by MDR pathogens have focused on epiphytic bacteria, which colonize the surface of leaves, roots, seeds, and fruits (5) and thus remain susceptible to disinfecting methods, which have been shown to be effective against bacteria colonizing fruit and vegetable surfaces (45). Therefore, identification of CTX-M-15-producing *Enterobacterales* with endophytic lifestyles is a critical public health issue, since endophytic bacteria colonize protected sites of internal plant tissues, from where they are able to resist conventional treatments used for disinfecting leafy vegetables (13, 46). Consequently, CTX-M-15 producers with endophytic lifestyles could begin to colonize hosts that use vegetables in the diet.

To support this hypothesis, we performed assays measuring tolerance to acid pH using TSB adjusted to pH 2, 3, 4, 5, 6, and 7 to define the survival of the endophytic CTX-M-positive *Enterobacterales* identified in this study. Low pH values were chosen in order to evaluate the ability to survive transit through the acidic conditions of the stomach, which is essential for successful colonization of the mammalian host by commensal and pathogenic bacteria (47). Interestingly, both *K. pneumoniae* and *E. coli* strains exhibited tolerance to acid pH, which was supported by the presence of *eefA* and *gad* genes. While the *gad* system helps to maintain a near-neutral intracellular pH when cells are exposed to extremely acidic conditions, *eefA* confers to the bacteria an acid tolerance response to inorganic acids (48).

Although, the origins of clinically relevant CTX-M-15-producing bacteria found in fresh vegetables in this study were not investigated, they could originate from human (as sewage), animal (manure and wild animal feces), and/or environmental (such as contaminated soil and irrigation water) sources that come into contact with crops (49–52). In this regard, colonization of vegetables can occur through entrances such as stomata, lenticels, root hairs, lesions, and emergent surfaces of radicle and lateral roots (53, 54). Additionally, animal and human pathogens, especially *E. coli* pathotypes, are also able to colonize endospheres (4). This last hypothesis could be supported by the endophytic properties and plant-colonizing abilities of CTX-M-15-producing strains, as observed in this study. In fact, using the common bean model, all strains exhibited endophytic colonization ability.

Another clinically relevant result of this study is the identification of endophytic CTX-M-15-producing *E. coli* strains belonging to pandemic high-risk clonal complexes CC38 and CC648, and *K. pneumoniae* of complex CC307, which have been associated with extraintestinal diseases and (mainly) bloodstream and urinary tract infections (BSIs and UTIs, respectively) (19, 55–58). In Brazil, these international clones have been

previously identified in human and animal infections and in polluted environments, denoting One Health implications (38, 59–63).

The wide host range of these critical priority clonal complexes, including different vegetables evaluated in this study, supports a genetic versatility and adaptation mediated by the gene content, which includes genes conferring endophytic properties and resistance to antibiotics, biocides, and heavy metals. In fact, an IncFIB plasmid (pKP301cro) coharboring the $bla_{CTX-M-15}$ gene and heavy metals resistance genes (i.e., *cusSRCFBA*, *copE2ABCDRSE1*, and *arsRDABC*) was identified. Heavy metals can come from contaminated soil, irrigation water, and inorganic fertilizers and pesticides commonly used in agricultural practices, which remain in the environment for long periods, accumulating in leaves, stem, and root of plants (45, 64–67). Consequently, these compounds, as well as biocides, may act as selectors of strains resistant to antibiotics. Therefore, the presence of multidrug-resistant pathogens displaying endophytic lifestyles and broad resistomes, resident in fresh vegetables, denotes environmental and food contamination mediated by anthropogenic activities. Future studies that include the analysis of a higher number of vegetables samples of different origins and the quantitative analysis of ESBL producers are worthy of further investigation, in order to gather data for risk assessment.

In conclusion, the occurrence of international clones of critical World Health Organization priority pathogens that are producing CTX-M-15 ESBL, harboring a broad resistome, and displaying endophytic lifestyles in fresh vegetables is a public and environmental health problem; it denotes contamination mediated by anthropogenic activities and a potential risk of human and animal exposure to antibiotic-resistant bacteria and/or their resistance genes. Therefore, fresh vegetables marketed for consumption can act as a figurative Trojan horse for the hidden spread of multidrug-resistant and ESBL-producing pathogens, which could be important bioindicators of environmental and food contamination.

MATERIALS AND METHODS

Isolation of endophytic bacteria displaying a broad-spectrum cephalosporin-resistant profile from surface-sterilized fresh vegetables. During a Brazilian surveillance study (OneBR project), conducted to characterize the burden of antimicrobial resistance associated with critical WHO priority pathogens, 48 samples of fresh vegetables collected from the São Paulo State Food Supply Company, the largest supply center in South America, were investigated. Vegetables included lettuce ($n = 6$), spinach ($n = 6$), escarole ($n = 6$), watercress ($n = 4$), beet ($n = 4$), arugula ($n = 4$), kale ($n = 4$), radish ($n = 4$), cabbage ($n = 4$), celery ($n = 2$), leek ($n = 2$), and chicory ($n = 2$). All samples collected were immediately stored in sealed plastic bags at 4°C and processed within 24 h. Samples were washed in running water and sanitized before the isolation of endophytic bacteria. For surface sterilization, ~4 g of leaves were immersed sequentially in 70% ethanol (1 min), sodium hypochlorite (2.5% chlorine, 4 min), and 70% ethanol (30 s), and then washed three times in sterile distilled water (68). Aliquots of the sterile water used in the final rinse were plated directly onto nutrient agar to confirm the sterilization protocol. For the isolation of ESBL-positive endophytic bacteria, surface-sterilized leaves were macerated in 12 ml of saline solution, serially diluted, and plated in triplicate on MacConkey agar supplemented with ceftriaxone (2 μg/ml). After 24 h of incubation at 37°C, colonies were picked from the selective plates, subcultured, and streaked to obtain pure cultures. Identification of isolates was performed using the Vitek 2 system (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing and phenotypic confirmation of ESBLs. Bacterial isolates were subjected to antimicrobial susceptibility testing by the disk diffusion method, whereas MICs were determined by Etest strips (bioMérieux, Marcy l'Etoile, France), with interpretative criteria according to CLSI (69, 70) or EUCAST (www.eucast.org). ESBL production was screened by the double-disk synergy test (71), with further confirmation by using Etest ESBL strips containing ceftazidime alone and in combination with clavulanic acid (bioMérieux, Marcy l'Etoile, France).

Whole-genome sequencing and bioinformatic analysis. All ESBL-producing endophytic isolates underwent whole-genome sequencing (WGS). For genome sequencing, total DNA was extracted from overnight cultures using the PureLink genomic DNA minikit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Sequencing was performed using the MiSeq platform (Illumina, San Diego, CA) (300 bp paired-end) and the reads were *de novo* assembled using SPAdes v.3.9 and A5-Miseq pipeline (72, 73). Sequence types (STs), serotypes, plasmid replicon types, antimicrobial resistance genes, and virulence genes were identified using MLST 2.0, SerotypeFinder 2.0, PlasmidFinder 2.1, ResFinder 4.1, and VirulenceFinder 2.0, respectively, available from the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/), and databases for bacterial genotyping from the Pasteur Institute (https://bigsdb.pasteur.fr/). Resistance genes with uncertain assignment by ResFinder were checked manually

and further blasted in NCBI. Analysis of transposable elements flanking bla_{CTX-M} genes was performed with ISfinder (74). For *E. coli*, virulence phylogroups were detected using the online Clermont typing tool (http://clermontyping.iame-research.center/). *K. pneumoniae* were further analyzed using Kleborate (https://github.com/katholt/Kleborate) to screen assemblies to confirm the species designation, multilocus sequence type (MLST), antibiotic-resistance genes, ICE*Kp*-associated virulence loci (yersiniabactin [*ybt*] and colibactin [*clb*]), and K (capsule) and O antigen (LPS) serotypes (75–77). Biocide-, heavy metal-, and disinfectant-resistance genes, along with genes to withstand acidic conditions, were identified using the BacMet-Scan script (http://bacmet.biomedicine.gu.se/) against the experimentally confirmed database v.2.0, using an E value = 1 and a threshold of >90% of identity and coverage.

Comparative phylogeny analysis of publicly available genomes of *E. coli* ST38 and ST648, from different countries, was performed using a minimum spanning tree constructed in Enterobase using the MSTree V2 algorithm and the wgMLST scheme (https://enterobase.warwick.ac.uk/species/index/ecoli), which consists of 25,002 pangenome genes present in *E. coli* genomes, representing most of the diversity in Enterobase at the time (February 2021) (https://enterobase.readthedocs.io/en/latest/pipelines/escherichia-statistics.html). Images were generated with iTOL v.5.5 (https://itol.embl.de). Comparative phylogeny of publicly available genomes of *K. pneumoniae* belonging to ST198 was performed using core-genome MLST (cgMLST) analysis and the BacWGSTdb database (http://bacdb.cn/BacWGSTdb/).

Conjugation and transformation of plasmids carrying ESBL genes. *E. coli* C600 (Str^R) and *E. coli* J53 (Az^R) were used as recipient strains in mating experiments with endophytic ESBL-producing *E. coli* strains as donors, in the ratio 3:1 (recipient:donor) in LB broth. Transconjugants were selected using MacConkey agar supplemented with ceftriaxone (2 μg/ml) and streptomycin (2,000 μg/ml), or ceftriaxone (2 μg/ml) and sodium azide (200 μg/ml). For transformation assay, plasmids were extracted by the alkaline lysis method (78) and ultracompetent *E. coli* TOP10 was heat shock transformed, as previously described (79), increasing the thermal shock time at 42°C to 1.5 min. Positive transconjugants and transformants were confirmed by ESBL production, as described above.

Endophytic properties of ESBL-producing *Enterobacterales*. Endophytic properties of ESBL-producing isolates were evaluated using a common bean (*Phaseolus vulgaris*) model (68), with modifications. In brief, bean seeds were surface sterilized and then incubated at 30°C until the early growth of the radicle (80). After two days of germination, sprouts were immersed for 30 min in the bacterial cell suspension (optical density at 600 nm [OD_{600}] = 1.5) and transferred to plant culture bottles with Murashige and Skoog medium, which were then incubated at 30°C for 15 days. Thereafter, bean plants were aseptically excised into root and shoot and endophytic bacteria were isolated from each of them, as described above. The recovered isolates were confirmed by detecting ESBL genes and by assessment of the clonal relatedness with the strains used to inoculate the sprouts, as determined by comparative enterobacterial repetitive intergenic consensus (ERIC)-PCR analysis (81). All assays were performed in triplicate. *Acinetobacter baumannii* ATCC 19606 and sterile distilled water were used as negative controls.

Tolerance of endophytic ESBL (CTX-M-15)-producing *Enterobacterales* to acid pH. Trypticase soy broth (TSB) culture medium was prepared to cover acid pH scales ranging from 2.0 to 7.0. Volumes of 50 ml of TSB were adjusted individually to a final pH of 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 by aseptically adding 1 N HCl, and using a pH meter (82). Broths were sterilized and the pH was confirmed. Next, 200 μl of each broth at the different pH values were added per well in a 96-well flat-bottomed microtiter plate. All endophytic ESBL producers were tested for pH tolerance (83). In brief, each well of the microtiter plate was inoculated with bacterial cell suspension to a final concentration of 10^5 cells per well and then the microplates were incubated at 35°C. After 1, 2, and 24 h of incubation, an aliquot (50 μl) of cell suspension was taken from each well, diluted 1:10, 1:100, 1:1,000, and 1:10,000, and cell viability was determined by plating 50 μl of each dilution on Trypticase soy agar (TSA) plates and incubating for 24 h at 35°C (84). All assays were performed in duplicate.

Statistical analysis. Data were subjected to analysis of variance (ANOVA) followed by the Duncan's multiple range test with a significance level of $P < 0.05$, using IBM SPSS Statistics 24 software (IBM, United States).

Data availability. Nucleotide sequences of endophytic CTX-M-producing *Enterobacterales* have been deposited in the GenBank database under accession numbers: PPHP01000000 (*E. cloacae* ESP151); MRWC01000000 (*K. pneumoniae* ALF301); PPHO01000000 (*K. pneumoniae* RUC232); PPHN01000000 (*E. coli* ESP110); PPHM01000000 (*E. coli* REP215); PPHL01000000 (*E. coli* REP237); KY354306.1 (plasmid pKP301b from *K. pneumoniae* ALF301); and KY495890.1 (plasmid pKP301cro from *K. pneumoniae* ALF301). Additionally, genomic data of *E. coli* ESP110, REP215, and REP237 and *K. pneumoniae* ALF301 and RUC232 strains are available on the OneBR platform (http://onehealthbr.com/) under numbers ID ONE110, ONE111, ONE112, ONE249, and ONE250, respectively.

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We declare there are no competing interests.

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