Characterization of the genetic environment of $bla_{ESBL}$ genes, integrons and toxin-antitoxin systems identified on large transferrable plasmids in multi-drug resistant Escherichia coli

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Objectives: Previously 14 conjugative plasmids from multi-drug resistant (MDR) *Escherichia coli* from healthy humans and food-producing animals in Switzerland were sequenced. The aim of this study was to extend the genetic characterization of these plasmids with a focus on $bla_{ESBL}$ genes including $bla_{CTX-M-1}$ and $bla_{TEM}$, class 1 integrons and toxin-antitoxin (TA) systems contained therein.

Methods: The nucleotide sequences and subsequent annotation therein of 14 conjugative plasmids were previously determined from their corresponding transconjugants. The TA loci were confirmed by RASTA-Bacteria.

Results: Eight of the conjugative plasmids identified were found to encode genes expressing ESBLs. Structural heterogeneity was noted in the regions flanking both the $bla_{CTX-M-1}$ and $bla_{TEM}$ genes. The $bla_{CTX-M-1}$ genes were associated with the common insertion sequences ISEcp1 and IS26, and uniquely with an IS5 element in one case; while $bla_{TEM}$ genes were found to be associated with IS26 and Tn2. A new $bla_{TEM-210}$ gene was identified. Seven class 1 integrons were also identified and assigned into 3 groups, denoted as In54, In369 and In501. Sixteen TA loci belonging to 4 of the TA gene families (relBE, vapBC, ccd and mazEF) were identified on 11 of these plasmids.

Conclusions: Comparative sequence analysis of these plasmids provided data on the structures likely to contribute to sequence diversity associated with these accessory genes, including IS26, ISEcp1 and Tn2. All of them contribute to the dissemination of the corresponding resistance genes located on the different plasmids. There appears to be no association between $\beta$-lactam encoding genes and TA systems.

Keywords: $bla$ genes, plasmid sequencing, CTX-M, TEM, accessory genes

INTRODUCTION

Understanding the molecular epidemiology of antimicrobial drug resistance genes has been a complex task due to the plasmids’ role in the spread of these elements (Carattoli et al., 2006; Hopkins et al., 2006). Moreover, the diversity and promiscuity of resistance genes has become a major global public health issue since antimicrobial resistance threatens the effective prevention and treatment of an ever-increasing number of infections in a clinical setting. Plasmid-located genes can be acquired from several sources and disseminated by horizontal gene transfer (HGT). The latter strategy contributes to the dissemination of many of the undesirable phenotypes associated with bacteria, including antibiotic resistance, virulence, and resistance to heavy metals (Davies and Davies, 2010; Baquero et al., 2013). Transmissible plasmids can be considered as particularly successful entities within the communal gene pool, and those that encode a full set of conjugation-encoding genes are referred to as conjugative, facilitating their dissemination over large taxonomic distances (Norman et al., 2009). Of note, there is a need to standardize surveillance methods with an emphasis on gene tracking by plasmid sequencing as an aid to reveal the transmission of resistance among bacteria from animals to humans vice versa.

*Escherichia coli* producing extended-spectrum $\beta$-lactamases (ESBLs) is a major problem in worldwide, since these ESBL genes can be spread by plasmid-mediated integrons, insertion sequence (IS) elements, and transposons among different bacteria species causing outbreaks as well as sporadic infections (Liebana et al., 2013). Plasmids expressing an ESBL phenotype frequently carry genes encoding resistance to other commonly used antimicrobial drug classes and genes for toxin-antitoxin (TA) systems, which contribute to the maintenance of plasmids in their host. Thus, understanding the roles of these genetic
elements and the surrounding genetic structures are essential and relevant to the subsequent development of strategies to limit the dissemination and persistence of ESBL genes and others.

In a recent publication (Wang et al., 2014), we reported the complete nucleotide sequences of 14 large (>30 kb) conjugative plasmids identified in nine multi-drug resistant (MDR) E. coli isolates expressing an ESBL phenotype isolated from food-producing animals and healthy humans in Switzerland. A comparative analysis of these plasmid-backbone structures was carried out previously (Wang et al., 2014). The aim of the present study was to analyse, in detail, the accessory genes from these plasmids for (i) the genetic context in which \textit{bla}_{\text{CTX-M-1}} and \textit{bla}_{\text{TEM}} genes were located, (ii) the structure of class 1 integrons and their gene cassettes, (iii) the identification of other related antimicrobial resistance genes and (iv) the identification of the TA systems.

**MATERIALS AND METHODS**

**BACTERIAL ISOLATES, SUSCEPTIBILITY TESTING AND GENETIC ANNOTATION**

Nine ESBL-producing \textit{E. coli} isolated from fecal samples of food-producing animals and healthy humans in Switzerland from 2009 to 2011 (Geser et al., 2012a,b), were identified previously. All of the associated technical protocols were described in earlier publications (Wang et al., 2013, 2014). The antimicrobial resistance profiles for these isolates are shown in a supplementary table (Table S1).

**BIOINFORMATICS SEARCH FOR TA PAIRS**

Putative TA systems were identified in the 14 conjugative plasmids using the RASTA-Bacteria program version 2.12 (http://genoweb1.irisa.fr/duals/RASTA-Bacteria/). A summary of the TA features associated with the plasmids is shown in Table 1.

**RESULTS AND DISCUSSION**

**COMPARISON OF THE GENETIC ENVIRONMENT OF BLA\textsubscript{CTX-M-1} GENES**

Eight \textit{bla}_{\text{CTX-M-1}} genes were found to be located within various genetic contexts in the present study. Either a complete or partial \textit{ISEcp1} gene was identified proximal to the resistance gene (Figure 1). A complete \textit{ISEcp1} gene was more often identified (Figure 1A), along with a partial ORF (denoted as \textit{Dorf477}) and a \textit{Dmrx} gene located distally in this arrangement. In three of the eight loci studied, a novel genetic arrangement was also noted wherein a truncated copy of \textit{ISEcp1} gene was identified (denoted as \textit{DISEcp1}) and which proximally flanked the \textit{bla}_{\text{CTX-M-1}} genes with an IS26 element being located further upstream (Figure 1B).

Further consideration of the eight \textit{bla}_{\text{CTX-M-1}} containing plasmids suggested that they could be divided into two groups based on their different disrupted insertion sequence elements. The first group consisted of five of the eight plasmids, including pC49-108, pC59-112, pC60-108, pH1519-88, and pH2291-112 (Figure 1A and Table 1). All 5 plasmids were typed as IncI1 and showed a high degree of similarity (99% at the nucleotide level) with the reference IncI1 plasmid R64 (Table 1) (Wang et al., 2014). With the exception of pH2291-112, a \textit{tnpA} gene of 1263 bp was found to be located upstream of the four \textit{bla}_{\text{CTX-M-1}} genes (Figure 1A). This was typical of the genetic organization previously identified in \textit{bla}_{\text{CTX-M-1}} containing plasmids, and the former DNA

### Table 1 Summary of the TA features associated with all 14 conjugative sequenced plasmids from healthy human and food-producing animal origin previously reported in Switzerland.

| Plasmid name | Accession no. | Plasmid size (bp) | Inc-type/pMLST | IS | Integron/Transposon | \(\beta\)-Lactamase(s) identified | Toxin-Antitoxin genes |
|--------------|---------------|------------------|----------------|----|---------------------|-----------------------------|-----------------------|
| pH2332-166  | JK484626      | 166,594          | IncFil-IncFilB | –  | In369               | TEM-1                       | vapC/vagC; relE/relB     |
| pH2332-107  | JK484627      | 107,386          | IncB/O         | IS26-\textit{AlISEcp1}; IS26 | –                          | CTX-M-1                    | relE/relB               |
| pH2291-144  | JK484628      | 144,925          | IncFil-IncFilB | –  | In369; Tn2          | TEM-1                       | vapC/vagC               |
| pH2291-112  | JK484629      | 112,671          | Inc1/ST3       | IS\textit{Ecp1}; IS5 | In54 | CTX-M-1 | none identified |
| pH1519-88   | JK484630      | 88,678           | Inc1/ST145     | IS\textit{Ecp1} | Tn2 | TEM-210; CTX-M-1 | none identified |
| pH1519-76   | JG48631       | 76,197           | IncFil         | –  | –                  | –                          | mazF/maze               |
| pH1038-142  | JG48634       | 124,875          | IncF-IncN/ST1  | IS26-\textit{AlISEcp1}; IS26 | In501 | TEM-1; CTX-M-1 | reI/reLB               |
| pC60-108    | JG48635       | 108,662          | Inc1/ST3       | IS\textit{Ecp1} | In54 | CTX-M-1 | vapC-1/vagC-1; vapC-2/vagC-2; vapC-3/vagC-3; ccdB/ccdA; relE/relB |
| pC59-153    | JG48636       | 153,231          | IncFilA-IncFilB | –  | –                  | –                          | reI/reLB               |
| pC59-112    | JG48637       | 122,330          | Inc1/ST3       | IS\textit{Ecp1} | In54 | CTX-M-1 | relE/relB               |
| pC49-108    | JG48638       | 108,660          | Inc1/ST3       | IS\textit{Ecp1} | In54 | CTX-M-1 | reI/reLB               |
| pC23-89     | JG48639       | 89,513           | Inc1/ST36      | –  | Tn2 | TEM-52 | vapC/vagC               |
| pL2-87      | JG48640       | 87,042           | IncB/O         | –  | –                  | –                          | reI/reLB               |
| pL2-43      | JG48641       | 43,265           | IncN/ST1       | IS26-\textit{AlISEcp1}; IS26 | –                          | CTX-M-1 | none identified |

–Feature not identified.
sequence was determined to be 100% identical to IS\textit{Ecp1} (accession no.: AJ242809) in all cases. IS\textit{Ecp1} was originally identified proximal to the plasmid-mediated cephalosporinase gene \textit{bla}_{\text{CMY-4}} in an \textit{E. coli} isolate identified in the United Kingdom (accession no.: AJ242809). This unusual IS element was also commonly found to be associated with several \textit{bla}_{\text{CTX-M}} genes (Figure S1) (Karim et al., 2001; Saladin et al., 2002; Poirel et al., 2003; Lartigue et al., 2004). IS\textit{Ecp1} elements are known to play a dual role, acting both as a transposase and ensuring the expression of the downstream ORF via a strong putative promoter (Poirel et al., 2003, 2005; Hossain et al., 2004; Jacoby, 2009). In the case of pH2291-112, unusually the locus between the transposon and the \(\beta\)-lactamase resistance gene was interrupted by an IS5 element (Figure 1A).

Analysis of the DNA downstream of the \(\text{bla}_{\text{CTX-M-1}}\) genes showed a varied gene organization. In the \textit{CTX-M-1} cluster, a partial sequence of 342 bp denoted as \text{orf477} was present in all structures studied and followed by a partially deleted \text{Dmrx} gene of 110 bp. This organization (IS\textit{Ecp1-\text{bla}_{\text{CTX-M-1}}-\text{Dorf477-\text{Dmrx}}}) was determined to be identical to the corresponding region found in other \textit{IncI} plasmid pCTX1261 (accession no.: HF549089) and also in an \textit{IncF} plasmid pCTX2412 (accession no.: HF549091) originally purified from \textit{E. coli} cultured from swine and horse samples in Germany (Schink et al., 2013). The data reported here further confirmed the importance of IS\textit{Ecp1} that is thought to function in the mobilization of \(\text{bla}_{\text{CTX-M}}\) genes among \textit{IncI1} type plasmids.

The insertion sequence element IS5 identified upstream of the \textit{bla}_{\text{CTX-M-1}} gene in pH2291-112 (Figure 1A) has only been reported on one other plasmid previously. The IS\textit{Ecp1-IS\textit{Ecp1}} arrangement was originally identified on a \textit{Klebsiella pneumoniae} plasmid p9701 (accession no.: FM246881) and this was associated with the mobilization of \textit{bla}_{\text{CMY-2}} gene (Figure S1B) (Verdet et al., 2009). Moreover, this arrangement was recently reported in the genomic DNA of \textit{E. coli} EC958, which was associated with one \(\text{bla}_{\text{CMY-23}}\) gene (Figure S1B) (Totsika et al., 2011).

The second group was unique in that a truncated copy of the IS\textit{Ecp1} transposon (denoted as \text{D\textit{Ecp1}}) was identified and found to be located between the \textit{bla}_{\text{CTX-M-1}} and an IS26. This unusual arrangement was identified in the remaining three plasmids: pH2332-107 (IncB/O), pH1038-142 (IncF-IncN), and pL2-43 (IncN) (Figure 1B). In all cases, the truncated D\textit{Ecp1} was 214 bp in length. All of these sequences contained the putative promoter region involved in the transcription of \textit{bla}_{\text{CTX-M-1}} genes. When the region proximal to the \textit{bla}_{\text{CTX-M-1}} gene was considered, it showed some unusual features. In the case of pH2332-107, the distal region of this gene consisted of the terminal 342 bp of \text{orf477}, followed by a partially deleted \text{Dmrx} gene (\text{Dmrx}), an entire \text{mph(A)} gene and a second copy of IS26 (Figure 1B). The gene module IS26-D\text{Ecp1}-\text{bla}_{\text{CTX-M-1}}-\text{Dmrx}-\text{mph(A)}-\text{IS26} was recently identified in plasmids purified from \textit{E. coli}, including those from the successful clone ST131 and from clinical \textit{E. coli} isolated from pigs in Germany (Cullik et al., 2010). In particular, this structure had been identified in several \textit{IncN} and \textit{IncI1} plasmids (Schink et al., 2011; Dolejska et al., 2013). Since the identical structure of the \textit{bla}_{\text{CTX-M-1}} region can be found in plasmids of different incompatibility groups, it has been hypothesized that the complete structure can be exchanged \textit{en-bloc} between different plasmid backbones as a composite transposon mediated by an IS26 transposition event (Cullik et al., 2010; Dolejska et al., 2013).

In contrast to the genetic arrangement above, in pH1038-142, the locus containing the \text{mph(A)} gene was deleted and extended beyond the distal IS26 with a region containing another gene, \text{bla}_{\text{TEM-1}} in addition to the \text{merA} and \text{merD} genes, encoding resistance to mercury (Figure 1B). Based on our extensive searching of the current databases, this is the first description of such an arrangement. Further examination of the \textit{bla}_{\text{CTX-M-1}} region in pL2-43 revealed other novel structural rearrangements. As shown in Figure 1B, similar to the situation described above for pH1038-142, the distal IS26 element, with intact left and right inverted repeats, was further extended with a copy of \text{mph(A)} followed by a hypothetical ORF (not shown in Figure 1B). These findings suggest that perhaps IS26 may play a hitherto unrecognized but important role in the mobilization of these ESBL genes.

**COMPARISON OF THE GENETIC ENVIRONMENT OF BLA\text{TEM} REGIONS**

Five \text{bla}_{\text{TEM}} genes were identified in this study. These were found to be located within three different genetic environments (Figure 2). In two of these (pH1519-88 and pH1038-142) the \text{bla}_{\text{TEM}} gene was located on the same plasmid containing a \textit{bla}_{\text{CTX-M-1}} gene. A complex transposon Tn2 was identified on three of these plasmids, pC23-89 (IncI1) containing \text{bla}_{\text{TEM}-22},
plasmids carrying \( \text{bla}_{\text{TEM-52}} \) within transposon Tn2, similar to that observed for pC23-89 (Figure 2A), have been described in *Salmonella enterica* and *E. coli* isolates from poultry and humans in Belgium, Germany, and the Netherlands, respectively, (Cloeckaert et al., 2007; Leverstein-Van Hall et al., 2011) and these appear to be widely distributed among different members of the Enterobacteriaceae family. Furthermore, a range of additional TEM variants (including TEM-20, −52, and −126) have similarly been detected in food-producing animals and from isolates cultured from foods in different European countries (De Champs et al., 2004; Sunde et al., 2009). Moreover, in relation to the ESBLs of the TEM class, the most frequently detected throughout the EU was reported to be TEM-52 [EFSA Panel on Biological Hazards (BIOHAZ), 2011].

Based on the sequencing data from pH1519-88, a novel TEM variant was identified and classified as \( \text{bla}_{\text{TEM-210}} \) (Figure 2A). Plasmid pH1519-88 also belonged to the IncI1 group. Sequence analysis of \( \text{bla}_{\text{TEM-210}} \) revealed two mutations which gave rise to two amino acid substitutions at positions corresponding to residues 49 and 69 when compared with a previously published sequence (accession no.: AF309824). The \( \text{bla}_{\text{TEM-210}} \) gene had also been identified within a Tn2 transposon (Figure 2A).

The Tn2-\( \text{bla}_{\text{TEM-1}} \) region of 4770 bp in length located on pH2291-144 exhibited 100% DNA sequence identity to that found in several plasmids in *E. coli*, including the conjugal plasmid pVQS1 (accession no.: JQ609357). The latter carries a *qnrS1* gene associated with an IncN group and was originally identified in a *Salmonella* Virchow isolate, which was cultured from a patient in Switzerland returning from a foreign holiday (Karczmarczyk et al., 2012). It is interesting to note that, whereas pH2291-144 and pH2332-166 showed 99% nucleotide identity across their backbones, the \( \text{bla}_{\text{TEM-1}} \) genes contained on these plasmids were associated with different transposons (Figures 2A,B). The \( \text{bla}_{\text{TEM-1}} \) gene of pH2291-144 was located within Tn2 and inserted within a \( \text{mer} \) module (Figure 2A), whereas in pH2332-166, the \( \text{bla}_{\text{TEM-1}} \) was flanked by two copies of IS26 (Figure 2B).

Several \( \text{bla}_{\text{TEM}} \) genes have been reported to be associated with different transposons, and with Tn2 in particular as the dominant \( \text{bla}_{\text{TEM}} \)-containing transposon in commensal *E. coli* (Bailey et al., 2011). Although information on the genetic context of the \( \text{bla}_{\text{TEM}} \) genes would be valuable in extending our understanding of how this important resistance gene is disseminated, for most TEM variants little or no sequence beyond the immediate flanking regions of the gene is available (Bailey et al., 2011).

**COMPARISON OF CLASS 1 INTEGRON REGIONS**

Several complete class 1 integron structures were noted among the 14 sequenced conjugative plasmids. Typically a class 1 integron is composed of three key features; a 5′-conserved structure (CS) containing an \( \text{intI1} \) site-specific recombinase; followed by a variable region containing one or more gene cassettes and then the 3′-CS containing the \( \text{qacE} \Delta 1 \) and a \( \text{sulI} \)-encoding genes. A complete class 1 integron was identified in plasmids pH2332-166 and pH2291-144 (Figure 3), wherein two gene cassettes \( \text{dfrA}1b-aadA1b \) were identified in the typical head-to-tail arrangement. Furthermore, on pH2332-166, three antimicrobial
resistance genes, strB, strA and sul2, that encode resistance to streptomycin and sulphonamide, were identified and found to flank the 5′-CS region of a complete class 1 integron; these latter genes were lacking on pH2291-144 (Figure 3). Sequence analysis of the segment (sul2-strA-strB-intI1-dfrA1b-aadA1a-qaCEDA1-sul1) (Figure 3) on pH2332-166 exhibited 100% similarity to the corresponding sequence located on the plasmid pNRG857c (accession no.: CP001856), which was previously isolated from a clinical isolate of adherent and invasive E. coli in Germany (Nash et al., 2010).

Located on pH1038-142 was the gene cassette containing dfrA1b-aadA1a and this was located distally to a 5′-CS which in this case was devoid of a complete 3′-CS, being replaced instead by a single copy of qaCeda2 and an insertion sequence element, IS440 (Figure 3). Downstream of the latter element was a sul3 gene that encodes resistance to sulphonamides. The cluster of intI1-dfrA1b-aadA1a-qaCeda2-IS440-sul3 shared 100% identity to the corresponding DNA sequence with plasmid pEC54 (accession no.: CP001856), which was previously isolated from a clinical isolate of porcine origin in Germany.

A similarly structured class 1 integron was also identified from the R64-like IncI1 plasmids, including pH2291-112, pH2291-144, pH2038-142, pH2291-112, pC49-108, pC59-112, and pC60-108. Areas shaded in gray and the arrangements of the ORFs are as indicated in Figure 2 above. IS elements are shown as arrowed boxes.

FIGURE 3 | Schematic presentation showing the complete class 1 integron along with the flanking regions identified on plasmids pH2332-166, pH2291-144, pH1038-142, pH2291-112, pH2291-144, pH1038-142, pH2332-166, pH2291-112, and pH1038-142 respectively (Figure 4 and Table 1). The loci (tnpA-pecM-tet(A)-tetR) showed a high degree of sequence similarity to each other, at 99% nucleotide identity. The tet(A) gene on each plasmid was flanked on the distal side by a pecM gene encoding a PecM-like protein, which has been reported previously to be associated with tetAR genes (Pasquali et al., 2005; Szczepanowski et al., 2011). On pH2332-166 and pH2291-144, the tetR genes were located on the distal side of a mercury resistance cassette (Wang et al., 2014). In plasmid pH1038-142 this module was located between a class 1 integron and a strA module. It has been suggested previously that the tetAR and adjacent genes serve as a hot-spot for insertion of different mobile elements and can be modified by direct-repeat-mediated deletional events (Szczepanowski et al., 2011). It is conceivable that additional resistance modules could be integrated into the tet-platform.

A sul2-strA-strB gene cluster was noted on pH2332-166 (Figure 3), encoding resistance to sulphonamide and streptomycin, which showed a high degree of similarity with that of plasmid pCERC1 (accession no.: JN012467), at 99% nucleotide identity. The plasmid pCERC1 carrying the dfrA14 cassette in the strA gene of the sul2-strA-strB gene cluster, has been reported in many countries, indicating a global distribution and it appears to have been circulating in Gram-negative bacteria for more than 25 years (Anantham and Hall, 2012).

All of the transconjugant strains were re-screened for antimicrobial resistance genes as previously described (Karczmarczyk et al., 2011). The antimicrobial resistance profiles for the nine strains in this study are shown in a supplementary table (Table S1).

TA SYSTEMS IDENTIFIED ON PLASMIDS ISOLATED FROM E. coli IN HEALTHY FOOD-PRODUCING ANIMALS AND HUMANS

TA loci play a role in bacterial stress physiology and the stabilization of horizontally acquired elements. TA systems are composed of a toxin-encoding gene along with its cognate antitoxin and these features prevent post-segregational killing by the host bacterial cell. In addition, these genetic loci also act to eliminate compatible plasmids, therefore ensuring the maintenance of the plasmid in the bacterial cell during replication (Hayes, 2003; Unterholzner et al., 2013). In adapted E. coli, it has been proposed, previously that strain virulence and the association with
Accessory genes from conjugative plasmids could contribute to the success and spread of the \textit{bla}_{CTX-M-15} genes (Clermont et al., 2008; Mnif et al., 2010). Furthermore, recent experiments reported that ESBL-plasmids carrying TA systems can be cured from field isolates of \textit{E. coli} using a heat technique, and the cured ESBL-plasmids contained at least one complete TA system, whose loss would normally mean the death of bacterial cells (Schaufler et al., 2013). TA loci-encoded toxins themselves may represent a novel target that could be exploited for the development of future generation of antibacterial compounds (Gupta, 2009).

Using BLASTP and RASTA-Bacteria, we searched all 14 conjugative plasmids and identified four different TA families (Table S2). Six TA loci, denoted as VapC (toxin protein) and VagC (antitoxin/virulence-associated protein), were identified on plasmids pH2332-166, pH2291-144, pC59-153, and pC23-89. The addiction module toxin RelE and antitoxin RelB protein were detected from eight plasmids, pH2332-166, pH2332-107, pH2291-112, pC60-108, pC59-153, pC59-112, pC49-108, and pL2-87, respectively. A representative of the \textit{mazEF} family was located on the multidrug resistant plasmid pH1038-142. The plasmid maintenance protein, toxin CcdB and antitoxin CcdA protein, were only detected on plasmid pC59-153. A summary of TA systems from the 14 conjugative plasmids and the corresponding protein sequences are shown in Table S2.

A phylogenetic tree containing the RelE/VapC/CcdB/MazF superfamily toxins identified on these plasmids in this study is shown in Figure 5. Four clusters containing these toxin sequences were noted. The RelE superfamily was located on plasmids with different Inc types in this study (Figure 5 and Table 1). In IncI1 plasmids, the RelE sequences were identical, with variations being evident from the clustering when located in plasmids of IncB/O or IncF types (Figure 5). It is also interesting to note that pC59-153 possessed three \textit{vapC} toxin-encoding genes, which were located in different plasmid genomes. Additionally, the backbone of pC59-153 showed high levels of nucleotide similarity with several CoIV plasmids that are known to be associated with an avian pathogenic \textit{E. coli} strains (Wang et al., 2014). Thus, the presence of multiple \textit{vapC} toxin-encoding genes and other TA systems in pC59-153 could support plasmid stability and virulence. Figure 5 shows that the six VapC sequences cluster into two different groups according to their amino acid sequence similarities, and all but one belonged to IncF types. While the relationship between the TA systems and the plasmid incompatibility types remains unclear, further study may help in the understanding of the nature of these TA systems in plasmids and their specific physiological role(s) in bacteria. Amino acid sequence similarity and clustering analysis of the antitoxin genes were carried out and these showed similar characteristics to their corresponding toxin genes (data not shown).

In many cases, TA loci were clustered or closely linked to mobile genetic elements (Pandey and Gerdes, 2005). In the most extreme of these cases, three \textit{vapC/vagC} loci from plasmids pH2332-166, pH2291-144, and pC59-153, were found to be located in the \textit{traD-traI} intergenic region. All \textit{relBE} homologous loci identified in our study, were \textit{bona fide} integron elements located between replication proteins and mobile element proteins. It is tempting to speculate that these features suggest that TA loci may represent a type of mobile cassette that can move between plasmids of different Inc types or different original bacterial sources and that undergo rapid evolution and horizontal transfer.

CONCLUDING REMARKS

In this study we extended our careful analysis of the accessory genes in 14 conjugative plasmids from nine unrelated human, poultry and lamb \textit{E. coli} isolates. These plasmids have the capacity

**FIGURE 5** | Phylogenetic tree (chladogram) for the amino acid sequences of plasmid-encoded toxin genes from 14 conjugative plasmids (see Table 1). The TA loci sequences were retrieved from Table S2 (raw sequences). The tree was calculated using Clustal Omega version 1.2.1. The lengths of the horizontal lines indicate relative evolutionary distances. A scale bar is also shown.
to transfer at high frequency to suitable recipient bacterial strains, providing a means by which the blaESBL genes harbored therein can be quickly disseminated. This feature may in turn compromise the use of this class of antibiotic for the treatment of infections in both animals and humans alike. Furthermore it suggests the existence of a reservoir of resistance genes that is highly mobile and contained in more than one genetic context. Our current study reports on some novel arrangements that were identified in the plasmids conferring resistance to β-lactam and other commonly-used antibiotics, and the structural heterogeneity associated with these regions flanking antibiotic resistance genes. Insertion sequences and transposons, such as IS elements, are shown as boxes with an arrow indicating their transcription orientation. The ORFs are shown as arrows, with the arrowhead indicating the direction of transcription. iSEcpT elements are shown as boxes with an arrow indicating the transcription orientation. (B) Comparison of the blaCTX-M-1 gene environment in plasmid pH2291-112 with the blaCMY-2 gene in plasmid p9701. Areas shades in gray indicate homologies in the corresponding genetic region on each plasmid. The ORFs are shown as arrows, with the arrowhead indicating the direction of transcription. iSEcpT and IS5 elements are shown as boxes with an arrow indicating their transcription orientation.

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