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Diversity of CTX-M-1-producing *E. coli* from German food samples and genetic diversity of the $bla_{CTX\text{-}M\text{-}1}$ region on IncI1 ST3 plasmids

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ABSTRACT

Antimicrobial resistance to cephalosporins is commonly mediated by extended-spectrum β-lactamases (ESBL) or plasmidic AmpC β-lactamases (pAmpC). In livestock $bla_{CTX\text{-}M\text{-}1}$ is the most frequently detected ESBL-encoding gene. As transmission to consumers through contaminated food is often proposed, this study characterized ESBL/pAmpC-producing *E. coli* collected from food samples. Therefore, samples from food products of animal origin and vegetables were screened for phenotypically resistant *E. coli* by selective cultivation. The ESBL genotype was confirmed for 404 isolates with the majority of them (n = 212) harboring the $bla_{CTX\text{-}M\text{-}1}$ gene. PFGE and MLST analyses as well as plasmid characterization were carried out for 89 isolates, selected under epidemiological aspects. In addition, 44 isolates were investigated by whole genome sequencing and/or sequencing of their plasmids on an Illumina Miseq platform.

MLST and PFGE indicated a diverse population of CTX-M-1-producing *E. coli* in German food samples with no spread of single clonal lineages. The majority of the isolates harbored the $bla_{CTX\text{-}M\text{-}1}$ gene on IncI1 plasmids. Frequently, the gene was associated with the IS*Ecp1* element and located on a ~100 kb IncI1 plasmid depicting the plasmid multilocus sequence type (ST) 3. The $bla_{CTX\text{-}M\text{-}1}$ gene and its flanking sequences were located within the shufflon of the type IV pilus region in diverse orientations.

In conclusion, dissemination of the CTX-M-1 β-lactamase within food samples of animal origin is driven by the transmission of a ~100 kb large IncI1 ST3 plasmid. Apart from conjugal transfer of IncI1 ST3 plasmids the transmission of the $bla_{CTX\text{-}M\text{-}1}$ gene might be further promoted through mobilization due to its location within a recombination hot-spot of IncI1 plasmids.

1. Introduction

The resistance of bacterial pathogens to antimicrobials is one of the major public health issues of the 21st century. Broad spectrum antibiotics are the first and second line treatment choice for bacterial infections. Cephalosporins, a group of β-lactam antibiotics which inhibit the cell wall synthesis, are widely used in human as well as veterinary medicine. Limited treatment options due to reduced sensitivity are a risk for human and animal health. Resistance to 3rd generation cephalosporins are frequently mediated by degrading enzymes called extended-spectrum β-lactamases (ESBL) or AmpC β-lactamases (AmpC). The ESBL- or AmpC-encoding genes are typically localized on plasmids (Bonnet, 2004; Carattoli, 2013). ESBL-encoding plasmids detected in the European Union (EU) commonly belong to the incompatibility groups (Inc) F, A/C, N, HI2, I1 and K (EFSA Panel on Biological Hazards, 2011). The bla_{ESBL} and bla_{AmpC} genes are often associated with mobile genetic elements, like transposons (integrons), and insertion sequences (IS). Thus, horizontal gene transfer by

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mobilization or conjugation has a major impact on the dissemination of β-lactam resistance in different strains and Enterobacteriaceae genera (Liebana et al., 2013).

In the EU approximately 21.1 tons of cephalosporins were sold in the veterinary sector in 2015 (EMA/184855/2017). ESBL-producing *E. coli* are found in food-producing animals and food of animal origin with up to 40% of the samples (EFSA (European Food Safety Authority) and ECDC, 2017). The CTX-M enzyme is the most commonly encoded ESBL enzyme type, with CTX-M-1 being the most frequently isolated subtype in samples of animal origin. The $bla_{CTX\text{-}M\text{-}1}$ gene is often located on IncI1 or IncN plasmids, which are highly self-transmissible (Dahmen et al., 2012; Moodley and Guardabassi, 2009). While IncN plasmids are broad host range plasmids, IncI1 plasmids are transferred only to a narrow host range (Götz et al., 1996; Pukall et al., 1996;). The IncI plasmids harbor several genes for formatting type IV pili. Besides motility and mating, type IV pili contribute to adhesion and invasion of Shiga-toxin *E. coli* (STEC) and other gram-negative pathogenic bacteria (Kim and Komano, 1997).

Transmission of ESBL-producing Enterobacteriaceae from animal to human is often postulated and food, especially retail meat is considered as a transmission vehicle. In a Dutch study, the occurrence of the $bla_{CTX\text{-}M\text{-}1}$ gene located on IncI1 plasmids in poultry, retail chicken meat and human isolates was demonstrated (Leverstein-van Hall et al., 2011).

In this study, $bla_{CTX\text{-}M\text{-}1}$ positive isolates from food were carefully characterized in regard to their genetic context, phylogenetic epidemiology and possible transmission pathways.

2. Methods, techniques

2.1. Samples and isolates

Within the German national research consortium RESET2, 2256 food samples (from poultry, pork, beef, milk, cheese and vegetables) were screened for cephalosporin resistant *E. coli* by selective isolation procedure (Irrgang et al., 2017). In short, 25 g from each sample were investigated by an unselective pre-enrichment step in lysogeny broth (LB) and subsequent selective cultivation on MacConkey Agar supplemented with 1 mg/l cefotaxime (CTX). Phenotypically resistant isolates were obtained from all types of sampled matrices, although only few isolates originated from vegetables. The samples were taken by official veterinarians and investigated in the federal states laboratories in the years 2012 and 2013. Only one phenotypically resistant isolate per sample was sent to the national reference laboratory for antibiotic resistance (NRL-AR), located at the German Federal Institute for Risk Assessment (BfR) for further investigations. In addition, some samples were taken, which couldn`t be taken into account of the epidemiological study because of limited information of the samples, but were investigated for the molecular analysis. The prevalence data will be published in detail separately.

The antimicrobial susceptibility for these isolates was determined by micro-broth dilution method. The antimicrobials tested were: ampicillin, chloramphenicol, cefotaxime, ceftazidime, ciprofloxacin, florfenicol, gentamycin, kanamycin, nalidixic acid, sulfonamide, streptomycin, tetracycline and trimethoprim. In addition, resistance to additional β-lactam antibiotics was investigated by disc diffusion method. The interpretation of the results was performed according to CLSI and EUCAST guidelines, respectively. The ESBL or pAmpC genotype was confirmed by PCR and sequencing (Irrgang et al., 2017). Only $bla_{CTX\text{-}M\text{-}1}$ harboring isolates were included in the study.

2.2. Molecular characterization

The phylogenetic group of each isolate was determined by PCR-amplification as previously described (Doumith et al., 2012). On the basis of samples origin and geographical region (as evenly heterogenic as possible distributed) as well as phylogenetic group, 89 isolates were selected and further characterized. As isolates of the phylogenetic group D are often associated with human infections, a larger proportion of these isolates from food were chosen for further analysis (Maamar et al., 2016). Thereof, XbaI restriction patterns were obtained by PFGE according to PulseNet protocol (https://www.cdc.gov/pulsenet/pathogens/protocols.html) as well as S1 nuclease PFGE for analyzing number and sizes of the plasmids (Irrgang et al., 2017). To determine which of the plasmids harbored the $bla_{CTX\text{-}M\text{-}1}$ gene, subsequent southern blot hybridization with a $bla_{CTX\text{-}M\text{-}1}$ probe was performed. PFGE analysis was assessed with Bionumerics 7.5 (Applied Maths, Sint-Martens-Latem; Belgium) including cluster analysis.

The sequence type of those isolates from which no whole genome sequence information were obtained (see below), was determined by PCR-sequencing using the Achtman's scheme (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search).

Conjugation of the $bla_{CTX\text{-}M\text{-}1}$-harboring plasmids into the *E. coli* strain ATCC87402 (pBAD3, cmr^R) was carried out by filter mating studies at 37 °C and selective cultivation of transconjugants on LB-agar containing 1 mg/l cefotaxime (CTX) and 100 mg/l chloramphenicol. The Inc group of the $bla_{CTX\text{-}M\text{-}1}$ plasmids was determined by PCR analysis of the transconjugants using the PBRT Kit (Diatheva, Cartoceto PU, Italy).

2.3. Sequencing and interpretation of sequence data

Whole genome sequencing (WGS) of 31 isolates which were selected to cover the different phenotypic patterns was carried out on the Miseq platform (Illumina, CA, USA). Therefore, genomic DNA was isolated from overnight cultures using the PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific, Schwerte, Germany) and a Nextera®XT library was generated. Furthermore, plasmids of 23 strains (selected independently from WGS) were also sequenced on a Miseq-Benchtop sequencer with 2 × 300 paired-end reads each. In total, sequence information was available from 44 isolates. The obtained raw reads were assembled either with the SPAdes algorithm (version 3.5.0) (Bankevich et al., 2012) or with the assembler of the CLC Genomics Workbench 9.5.2 (QIAGEN Bioinformatics). Information regarding MLST, plasmid content, pMLST, acquired resistance genes and virulence factors were obtained using the web-based tools (MLST, PlasmidFinder, pMLST, ResFinder, VirulenceFinder) provided by the Center for Genomic Epidemiology (http://www.genomicepidemiology.org) of the Danish Technical University (Larsen et al., 2018; Zankari et al., 2012; Carattoli et al., 2014; Joensen et al., 2014)

For SNP analysis whole genome data were processed and interpreted with Bionumerics 7.5 (Applied Maths). Raw reads first underwent a length trimming (settings: 300 bases; minimum sequence length: 20, maximum homopolymer length: 20 bases), followed by a quality trimming (minimum quality: 5; average quality: 20). For SNP analysis SNP filters for absolute coverage, relative coverage, unreliable bases, ambiguous bases, non-informative SNPs and inter-SNP distance were applied with default parameters. Annotation of $bla_{CTX\text{-}M\text{-}1}$ related contigs was carried out by PATRIC web resources (Version 3.4.13; www.patricbr.org). Alignment of the $bla_{CTX\text{-}M\text{-}1}$ region was performed using MAUVE 2.4.0 software (Darling et al., 2004).

3. Results

This study based on the screening of 2256 food samples (from poultry, pork, beef, milk, cheese and vegetables) for cephalosporin-resistant *E. coli*. A total of 437 phenotypically resistant isolates were obtained. An ESBL or pAmpC genotype was confirmed for 404 of these isolates (Table 1). The majority (n = 212) of them harbored a CTX-M-1 β-lactamase. CTX-M-1 positive *E. coli* were isolated from all types of analyzed food matrices with the largest proportion from poultry (46%; chicken, turkey), followed by samples of cattle origin (27%; beef, milk and cheese; Fig. 1A). The main part of the isolates belonged to

Table 1
Number of food samples investigated for the presence of ESBL/pAmpc producing *E. coli* in the epidemiological study according to the food matrix. Additional samples were investigated and the total numbers are given in brackets.

	No of samples taken in the epidemiological study	No of ESBL/pAmpC positive samples of the epidemiological study (all samples)	No of CTX-M-1 producing *E. coli* isolates epidemiological study (all isolates)
Poultry meat (chicken, turkey)	426	206 **(254)**	80 **(97)**
Pork	498	63 **(63)**	48 **(55)**
Beef	527	35 **(43)**	26 **(31)**
Milk (including raw milk) and cheese	406	36 **(39)**	28 **(28)**
Vegetables	399	1 **(1)**	1 **(1)**
Σ	2256	341 (**404**)	182 **(212)**

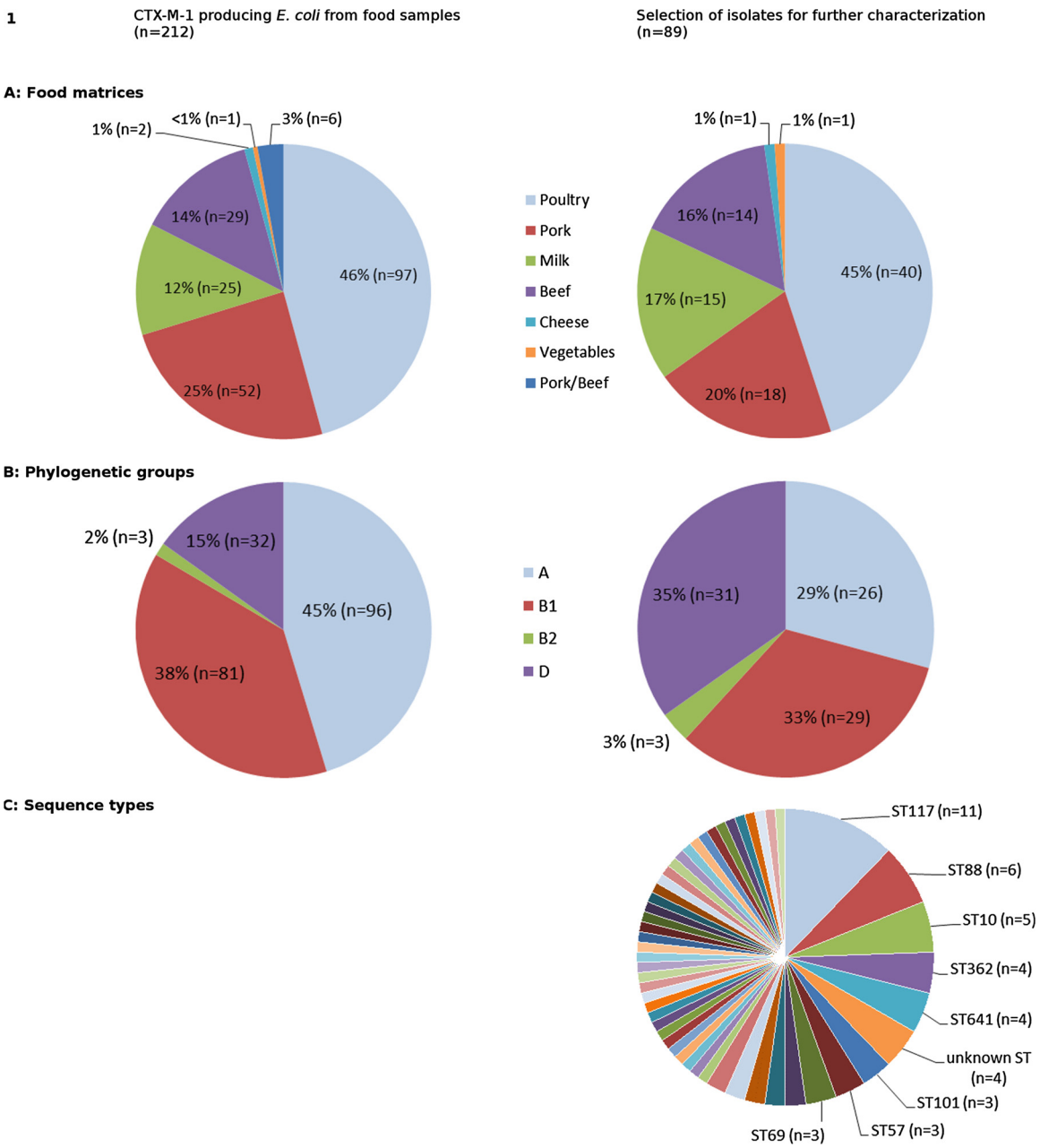

Fig. 1. A: Overview of the food matrices, where the CTX-M-1 producing *E. coli* were isolated from; all isolates (left column) and selection for characterization (right column). **B**: Phylogenetic groups of all detected $bla_{CTX\text{-}M\text{-}1}$ positive isolates (left) and selection for further investigations (right). **C:** Diversity of 51 different sequence types (ST) detected within 90 *E. coli* isolates. Only ST's which were detected at least three times were labeled for clear presentation.

phylogenetic groups A and B1 (45% and 38%). Only two isolates (2%) belonged to phylogenetic group B2, whereas 15% were assigned to phylogenetic group D (Fig. 1B). Based on defined criteria 89 representative isolates were chosen for further characterization (Fig. 1A and B).

MLST and PFGE analyses revealed a high diversity of CTX-M-1-positive isolates in German food samples. Among the 89 deeper characterized isolates, 51 different ST-types were detected. The most abundant type was ST117 (n = 11) followed by ST88 (n = 6) and ST10 (n = 5). The clonal complex CC10 (ST10; ST34, ST48, ST167, ST617) was detected nine times (Fig. 1C). All isolates of ST117 belonged to phylogenetic group D, whereas almost all isolates of ST88 and isolates of the CC10 belonged to phylogenetic group A. Five out of the nine CC10 isolates were isolated from raw milk or cheese from raw milk. In contrast, all but one isolate of the ST117 group originated from poultry meat. The XbaI PFGE analyses revealed a close phylogenetic relationship only for the two ST117 isolates RL323 and RL426. There was no obvious close phylogenetic relationship observed for the remaining 87 analyzed strains. The comparison of XbaI-PFGE restriction patterns of isolates from food samples of this study with isolates obtained from livestock within the same period revealed no clonal relationship between these two sample pools (data not shown). Hence, cluster analysis revealed no separation of isolates from animal and food samples. In comparison, a SNP analysis was performed with WGS data of 31 isolates. The cluster analysis revealed five independent clades. Within these clades, isolates of the same sequence type or clonal complex clustered together (Fig. 2). The described results, including acquired resistance genes or virulence genes, are summarized in Table S1.

Using S1 nuclease PFGE and southern blot hybridization, it was shown, that 40 strains (44%) harbored the $bla_{CTX\text{-}M\text{-}1}$ gene on a ~100 kb (± 6 kb) plasmid. PCR analysis of the transconjugants revealed that the $bla_{CTX\text{-}M\text{-}1}$ gene was associated with an IncI1 replicon in 60 of the 89 isolates, including all plasmids with a size of ~100 kb. The pMLST analysis could be performed for 22 of the ~100 kb IncI1 plasmids (sequence data was available) and all but one isolate (RL158 with ST7) harbored the $bla_{CTX\text{-}M\text{-}1}$ on an IncI1 ST3 plasmid. An additional IncI1 ST3 plasmid with the size of ~115 kb was identified for isolate RL126. Other IncI1 pMLST-types were found to be ST58 (n = 2; 80 kb) and ST10 (n = 1; 200 kb).

The IncI1 plasmids were highly similar to the IncI1 reference plasmid R64 (GenBank Accession: AP005147.1). Investigation of genetic environment of the $bla_{CTX\text{-}M\text{-}1}$ gene in the plasmids backbone revealed the association of the $bla_{CTX\text{-}M\text{-}1}$ gene with IS*Ecp1* elements for the majority (n = 67) of isolates including all ~100 kb IncI1 ST3 isolates. The integration site of 16 of the IncI1 ST3 plasmids was investigated. The gene cassette for transposase – CTX-M-1 β-lactamase – tryptophan synthase was integrated in the IncI1 conjugative transfer region of the plasmids with 13 different variants being identified. Fig. 3 summarizes the diversity of the resistance gene region, where an 8523 bp sequence of the $bla_{CTX\text{-}M\text{-}1}$ with its flanking region of each of the 16 strains was aligned using progressiveMAUVE. Recombination of three fragments occurred between the genes for shufflon specific recombinase and the PilV protein (part of the type IV pilus). The sequences at the end of the fragments overlapped partly and contained a conserved sequence (5′-GTGCCAATCCGGTACN(N)TGG-3′), which is characteristically for the shufflon rearrangement site. Conjugation itself was not affected by the insertion of the resistance gene into the transfer region of the plasmid as conjugation experiments were successful.

4. Discussion

In this study, ESBL-producing *E. coli* could be isolated easily from food of animal origin. The CTX-M-1 β-lactamase is the most common ESBL in animals, so it is consistent that more than 50% of investigated ESBL-producing *E. coli* harbored the $bla_{CTX\text{-}M\text{-}1}$ gene (EFSA (European Food Safety Authority) and ECDC, 2017).

The recovered isolates belonged to a large extend (more than 80%) to the phylogenetic groups A and B1, which are associated with multiple antibiotic resistance. In contrast, human pathogenic *E. coli* are mainly associated with phylogenetic groups B2 and D (Clermont et al., 2000; Chakraborty et al., 2015). These isolates are often characterized through higher virulence but least antibiotic resistance. Nevertheless, $bla_{CTX\text{-}M\text{-}1}$ plasmids are highly conjugative and transmission to pathogenic bacteria poses a risk for human health (Carattoli, 2013).

MLST revealed 51 different sequence types. Such diversity was shown for isolates from the United Kingdom, the Netherlands and Germany of different sources before, as well as the more frequently isolation of ST10, ST88 and ST117 (Day et al., 2016). The correlation between ST117, which was the most abundant sequence type detected in this study and the phylogenetic group D seems to be typical for *E. coli* (Rodrigues et al., 2015; Cristóvão et al., 2017). A German hospital study on the occurrence of ESBL showed a high diversity of CTX-M-1-producing *E. coli*, too. Here, the most abundant sequence type was ST101, followed by ST58, and ST10 and ST453 with the same abundance (Gerhold et al., 2016). In another study from German patients, ST10 was found most abundant, followed by ST131 (Pietsch et al., 2017). So, other than in food, ST117 was not reported for infected humans in Germany, but ST10 (second most abundant in food) was detected frequently in human patients as well as in healthy and diseased animals (Schink et al., 2013; Gerhold et al., 2016; Pietsch et al., 2017).

There was a contrast between the results of the cluster analyses of XbaI restriction patterns and whole genome sequences. Whereas XbaI-PFGE revealed almost no close phylogenetic relationships between the isolates, same sequence types were found to be in the same clusters within the SNP tree. This is consistent with the report that PFGE seems to be more useful for identification of clones but less efficient for deeper phylogenetic analysis (Deng et al., 2015).

Data presented in this study have shown that in Germany resistance to cefotaxime mediated by CTX-M-1 β-lactamase in *E. coli* from food is more related to the dissemination of ~100 kb IncI1 plasmids than to the spread of certain clonal lineages of *E. coli*. All but one of these plasmids were classified by pMLST to ST3. This plasmid sequence type is known to be linked to the dissemination of $bla_{CTX\text{-}M\text{-}1}$ in Enterobacteriaceae from humans, livestock as well as companion animals in other European countries (García-Fernández et al., 2008; Carattoli, 2011; Dahmen et al., 2012). The high abundance of this plasmid type in this study population might reflect the effective transmission of the plasmid among Enterobacteriaceae also in German livestock. In addition, comparison with isolates from livestock populations did not hint towards a clonal transmission of isolates from livestock to food. This underlines the hypothesis that transmission of $bla_{CTX\text{-}M\text{-}1}$ is mainly linked to horizontal gene transfer of mobile genetic elements (Zurfluh et al., 2014).

Our IncI1 ST3 plasmids showed high similarities within the backbone structure to the IncI1 reference plasmid R64 (GenBank Accession: AP005147.1). The CTX-M-1 region (IS*Ecp1* - CTX-M-1 – tryptophan synthase) integrated downstream to the shufflon specific recombinase gene, whereas recognition sequence of Rci recombinase, some *pilVB* and *pilVC* segments and some shufflon repeats were deleted of the plasmid R64 during integration. This region was formed through clustered inversion (called “shufflon”) (Komano et al., 1987) and is located next to genes encoding for conjugation transfer proteins (upstream) and proteins formatting type IV pilus (Sampei et al., 2010). The high diversity of DNA arrangement of the $bla_{CTX\text{-}M\text{-}1}$ integration site supports the hypothesis that this shufflon region is a hotspot for DNA rearrangement (Komano et al., 1986). The conserved sequence described by Komano et al. (1987) where the crossover of the recombination occurs, was identified in our strains within the flanking region of the divers arranged fragments (Komano et al., 1987). The inverted repeat sequences CCGG and GTAC might function as a recognition site. The structure of the shufflon and the meaning of the pilus type IV for adhesion of toxigenic *E. coli* are well-known. Integration of the $bla_{CTX\text{-}M\text{-}1}$ gene

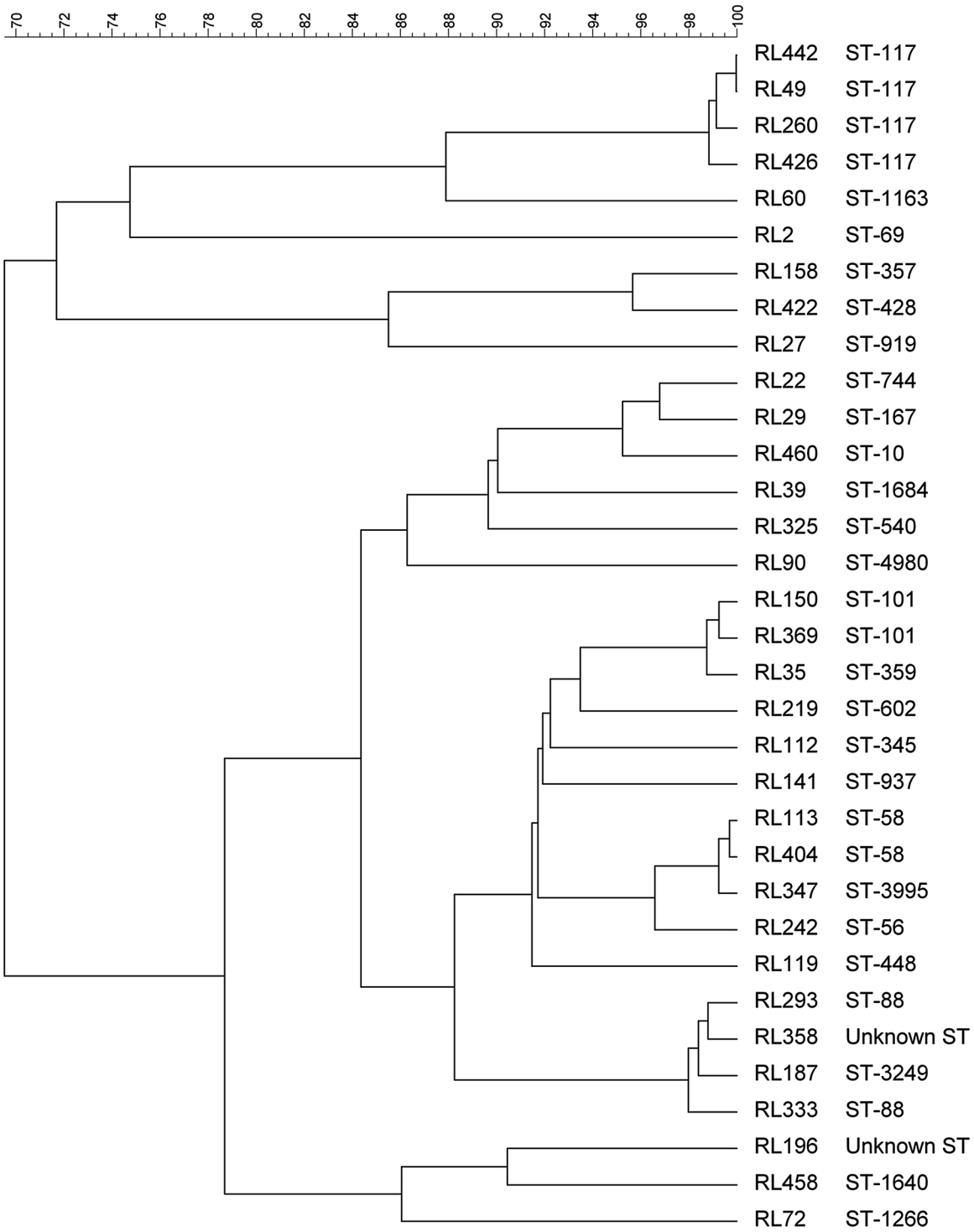

Fig. 2. Phylogenetic analysis of 33 CTX-M-1 producing *E. coli* obtained from food samples. SNP analysis was performed with Bionumerics 7.5 using UPGMA method and *E. coli* K12 MG1655 (NC_000913.3) as references. The scale defines the degree of the phylogenetic relationship of the isolates.

within this region was also shown previously for IncI1 plasmids and seems to be characteristic for ST3 plasmids (Smith et al., 2015). As the insertion region is defined as a high mobile DNA segment, transmission of the $bla_{\text{CTX-M-1}}$ gene might occur independently from conjugation of the Inc1 plasmid. In addition, the mobilization of the resistance gene is mediated by the association with the IS*Ecp1* (D'Andrea et al., 2013). We found this IS element in 67 of the 89 strains investigated. It was previously shown that IS*Ecp1* is also responsible for capture of $bla_{\text{CTX-M}}$ genes and has influence on the level of $bla_{\text{CTX-M}}$ gene expression (Ma et al., 2011).

5. Conclusion

This study was conducted within the project RESET and in the context of several studies with the aim to identify possible transmission routes between livestock and their environment, food and human. The CTX-M-1 β-lactamase could be found in more than 50% of the overall detected ESBLs in *E. coli* obtained from food samples. A clonal transmission from livestock to food or the spread of single clones within the reservoir food was not observed. The dissemination of CTX-M-1 β-lactamase in German food is highly related to the spread of IncI1 plasmids

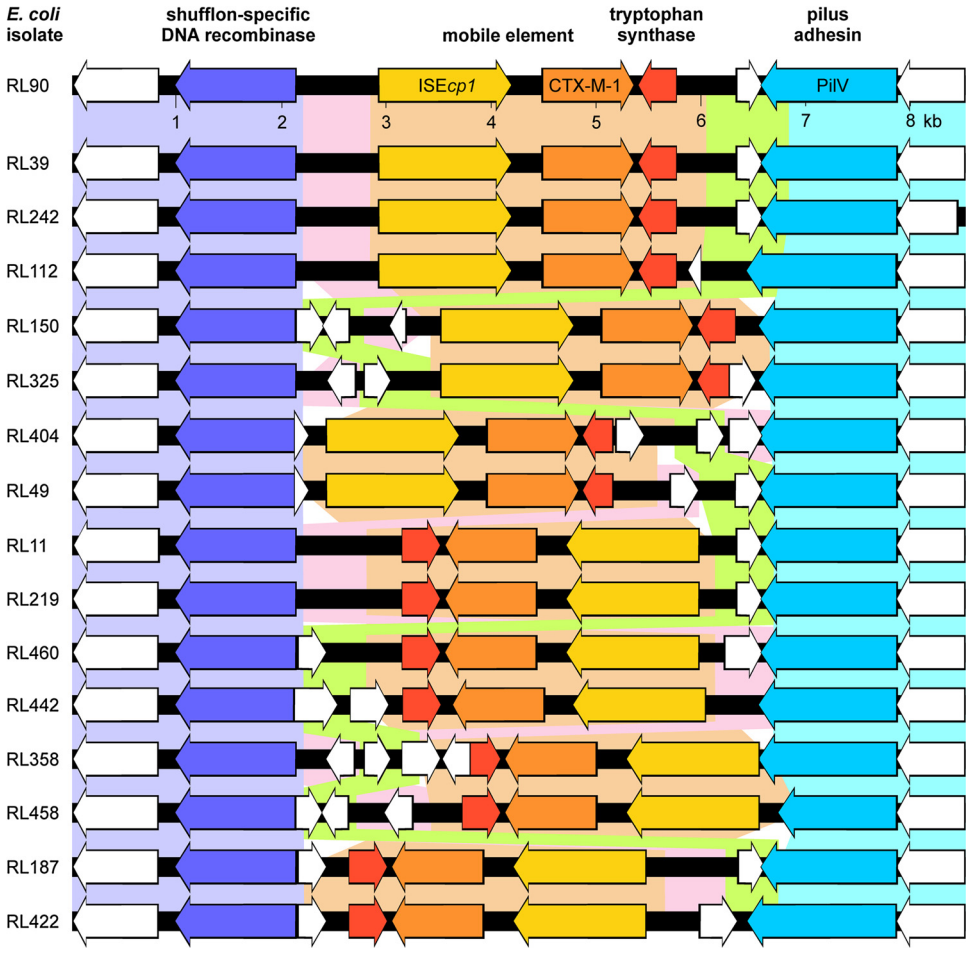

Fig. 3. Comparison of the $bla_{CTX\text{-}M\text{-}1}$ insertion site of 16 *E. coli* isolates harboring the gene on an IncI1 ST3 plasmid. Alignment was created using progressiveMAUVE and adjusted by CorelDraw Graphic Suits 3.0 for better interpretation. Relevant CDS (arrows) were labeled by their protein function in the sequence of the strain RL90 based on PATRIC annotation. Identical sequence sections are colored equally. White arrows indicating hypothetical proteins.

with most of them belonging to the ST3 with the size of ~100 kb.

Conflict of interest statement

None.

Disclaimer

B. Guerra is currently employed with the European Food Safety Authority (EFSA) in its BIOCONTAM Unit that provides scientific and administrative support to EFSA's scientific activities in the area of Microbial Risk Assessment. The positions and opinions presented in this article are those of the authors alone and are not intended to represent the views or scientific works of EFSA.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.06.003.

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