Originally published as:

Cullik, A., Pfeifer, Y., Prager, R., Von Baum, H., Witte, W.
A novel IS26 structure surrounds $_{bla}$CTX-M genes in different plasmids from German clinical Escherichia coli isolates
(2010) Journal of Medical Microbiology, 59 (5), pp. 580-587.

DOI: 10.1099/jmm.0.016188-0

A novel IS*26* structure is surrounding *bla*$_{\text{CTX-M}}$ genes in different plasmids of German clinical isolates of *Escherichia coli*

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Running title: CTX-M in Germany

Subject category: Clinical microbiology and virology

Accession numbers: The GenBank accession number for the newly determined sequence of *bla*$_{\text{CTX-M-65}}$ is EF 418608. The accession numbers for the *bla*$_{\text{CTX-M}}$ surrounding sequences in isolates 409, 390, 394, 396, 398, 404, 405, 406 and 407, respectively, are available at GenBank GQ274927 to GQ274935.

Summary

This report focuses on the molecular characterization of 22 extended-spectrum beta-lactamase (ESBL) producing *E. coli* isolates collected in a German university hospital, during a period of nine months in 2006. Relationship analysis of clinical isolates was done via pulsed-field gel-electrophoresis, multi locus sequence typing, plasmid profiling and additionally PCR for bla_{ESBL} detection and phylogroups. After conjugal transfer plasmid isolation and subsequent PCR for bla_{ESBL} detection and incompatibility groups were performed. Using one-primer-walking, up to 3600 bp upstream and downstream of different $bla_{\text{CTX-M}}$ genes could be sequenced. Beta-Lactamases found were TEM-1 (n=14), SHV-5 (n=1) and a wide variety of CTX-M types (n=21) as CTX-M-15 (n=12), CTX-M-1 (n=4), CTX-M-14 (n=2), CTX-M-9 (n=1), CTX-M-3 (n=1) as well as one new type CTX-M-65 (n=1). In 18 isolates bla_{ESBL} genes were located on conjugative plasmids in sizes between 40 and 180 kbp belonging to incompatibility groups FII (n=9), N (n=5) andI1 (n=4). Thereby $bla_{\text{CTX-M}}$ were found to be associated with the commonly known elements IS*Ecp1*, IS*26* and IS*903-D*, but with unusual spacer sequences for IS*Ecp1* in two isolates. These insertion sequences connected to $bla_{\text{CTX-M}}$ as well as other genes were located between two IS*26* elements in a configuration that has not been described yet. The results reveal the emergence of bla_{ESBL}, preferentially $bla_{\text{CTX-M}}$, located on different plasmids harboured by genotypic different *E. coli* strains. The identical gene arrangement in $bla_{\text{CTX-M}}$ neighbourhood in plasmids of different incompatibility groups indicates a main role of IS*26* in distribution of mobile resistance elements between different plasmids.

Introduction

Resistance to extended spectrum β-Lactam antibiotics is mainly caused by extended spectrum β-Lactamases such as *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ (Paterson & Bonomo, 2005). CTX-M type ones seemed to be particularly successful in terms of spread. Since its first description in 1989 86 variants have been found up to date (www.lahey.org/studies/). They are clustered in five subgroups (1, 2, 8, 9, 25) according to their amino acid homology (Tzouvelekis *et al.*, 2000; Bonnet, 2004). As natural reservoir chromosomal genes of different *Kluyvera* species have been identified. The natural diversity of CTX-M types is also found among nosocomial isolates which leads to the conclusion that the *bla*$_{CTX-M}$ genes have been picked up by different events (Rodriguez *et al.*, 2004). A number of genetic mechanisms have apparently been involved in aqusition of CTX-M genes. Insertion sequences IS*26*, IS*Ecp1* and IS*CR*1 in association with class 1 integron structures, as well as phage related elements seem to have played a prominent role in these processes (Arduino *et al.*, 2002; Eckert *et al.*, 2006; Oliver *et al.*, 2005; Poirel *et al.*, 2008). Moreover IS*Ecp1* elements and its remnants constitute an alternative promoter region (Karim *et al.*, 2001) which leads to increased, clinically relevant expression of the *bla*$_{CTX-M}$ gene that is only weakly expressed in its natural reservoirs (Karim *et al.*, 2001; Poirel *et al.*, 2003).

In nosocomial isolates *bla*$_{CTX-M}$ genes are mostly located on large plasmids ranging in size between 40 and over 200kb (Kariuki *et al.*, 2001; Saladin *et al.*, 2002; Pai *et al.*, 2001). They belong to a wide variety of incompatibility groups (Inc groups) mostly IncF, I, N, P and H, but IncA/C and L/M have also been found (Garcia *et al.*, 2007; Novais *et al.*, 2007; Diestra *et al.*, 2009). A large number of them are conjugative facilitating intra- and interspecies spread. Here we report pheno- and genotypic analyses of a collection of ESBL producing *E. coli* strains in a university hospital. We elucidate the *bla*$_{CTX-M}$ environment in selected isolates concerning different CTX-M types on plasmids of different incompatibility groups. The

analysis of the genetic environment of $bla_{\text{CTX-M}}$ genes can reveal details of acquisition with regard to their origin and further dissemination.

Material and Methods

Bacterial strains. During a period of nine months from January to October in 2006 22 *Escherichia coli* (*E. coli*) strains, that exhibited resistance to β-Lactam antibiotics, were collected in a German university hospital, assuming continuity. The strains isolated from urine (59%), tracheal secretions (14%), sputum (9%), wounds (9%) and faecal smears (9%) were all from different patients showing infections and being hospitalized in urology (35%), surgery (27%) as well as several other wards (38%). Patients were between three weeks and 87 years old, (51 years on average), 50% were male and female, respectively. Eleven patients were treated with fluoroquinolones and/or β-Lactam antibiotics previously.

Antimicrobial susceptibility testing. Standard microbroth dilution assay, according to CLSI protocol, was performed and resistance to 17 commonly used antibiotics belonging to different antibiotic classes was assessed (AMP, ampicillin; MEZ, mezlocillin; MSU, mezlocillin-sulbactam; CTM, cefotiam; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; AMK, amikacin; STR, streptomycin; NAL, nalidixic acid; CHL, chloramphenicol; OTE, oxytetracycline; CIP, ciprofloxacin; SMZ, sulfameracin; SXT, sulfameracin-trimethoprim) (CLSI, 2006). Phenotypical identification of ESBL producers was performed in a second, confirmatory microbroth dilution test detecting the resistance to three third generation cephalosporins (CTX, cefotaxime; CAZ, ceftazidime; CPD, cefpodoxime) in presence and absence of clavulanic acid (National Committee for Clinical Laboratory Standards, 1999; National Committee for Clinical Laboratory Standards, 1997).

Clonal characterization of *E. coli* isolates. PFGE was performed following the protocol of (Hunter *et al.*, 2005). TIFF files were analysed using the BioNumerics software.

Similarity values were computed using DICE coefficient and visualized in a dendrogram based on the UPGMA. Strains showing ≥ 90 % similarity were classified as genetically related and assigned to the same lineage.

All isolates were further analyzed by multilocus sequence typing (MLST) following the official protocol of the MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli/documents/primersColi_html).

The phylogenetic groups of these isolates were determined by a previously described PCR-based method (Clermont *et al.*, 2000). If not described otherwise, all PCR reactions in this study were done, using the illustra Ready to go PURE Taq beads (Amersham Biosciences) according to the manufacturer's instructions.

ESBL identification. The resistance genes for ESBL (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$) were amplified by multiplex-PCR and subsequently sequenced using the primers formerly described (Grobner *et al.*, 2009). 0,4 µL of CTX-M primers, 0,4 µL of SHV primers, 0,3 µL of CTX-M-9 primers and 0,9 µL TEM primers (c = 10 pmol μL^{-1}) were used. After an initial denaturation of 96 °C for 2 min, the protocol consisted of 30 cycles at 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s followed by a final extension at 72 °C for 7 min.

Sequencing. Sequencing reactions were performed with BigDye ® Terminator v3.1 Cycle Sequencing Ready Reaction Kit and run on ABI capillary sequencer. The nucleotide sequences were analyzed with Lasergene software and compared with data submitted to NCBI sequence database using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/blast/).

Plasmid analysis. Transfer of $bla_{CTX\text{-}M}$ carrying resistance plasmids was performed by broth mating assays using a sodium azide resistant *E. coli* J53 recipient. Transconjugants were selected on LB agar plates containing sodium azide (300 mg L^{-1}) and cefotaxime (5 mg L^{-1}) as performed by Jacoby & Han (Jacoby & Han, 1996).

Plasmid DNA of donor and transconjugants were isolated using the Plasmid Mini Kit (QIAGEN) and analysed on 0,4 % agarose gels using *E. coli* V517 and *E. coli* R27 as size

marker (Sherburne *et al.*, 2000; Macrina *et al.*, 1978). Plasmids obtained by conjugation were designated pKC and pKCT, respectively. Numbers were chosen according to isolate number.

PCR for determination of integron classes and incompatibility groups were performed as previously described by Mazel *et al.* (2000) and (Carattoli *et al.*, 2005; Mazel *et al.*, 2000), respectively using DNA from plasmid mini preparation of transconjugants and whole genomic DNA of the recipient strain as negative control.

Genetic environment of *bla*$_{\text{CTX-M}}$. Integron association of *bla*$_{\text{CTX-M}}$ genes was determined by long PCR using the DyNAzyme ™ EXT PCR Kit according to the manufacturer's instructions. Primers are listed in Tab. 1.

For elucidating the genetic environment of *bla*$_{\text{CTX-M}}$ genes walking PCR was performed accordingly to (Pilhofer *et al.*, 2007) using primers and annealing temperatures listed in Tab. 1. Furthermore primers were designed based on sequencing of the entire plasmid pKC394 (unpublished data). DNA samples were used as described above (plasmid analysis).

Confirmation of newly explored sequences accompanying *bla*$_{\text{CTX-M}}$ genes was performed by PCR using primers and annealing temperatures listed in Tab. 1. PCR conditions were chosen as described above (ESBL identification).

Cloning experiments. Relevant amplicons, obtained by one-primer-walking, that were present in transconjugants but absent in the recipient, were processed using a Gel Extraction Kit. The isolated fragments were subsequently ligated into a pCR®2.1 vector and transformed into chemical competent *E. coli* K12 DH5α TOP10F´ using the TA Cloning® Kit according to the manufacturer's instructions. Plasmid inserts were amplified using M13 primers. When showing expected sizes inserts were sequenced and analysed as described above.

Results and Discussion

137 The investigations on the ESBL producing *E. coli* isolates collected in a German
138 hospital in 2006 answer the questions, whether there is one circulating *E. coli* clone or
139 dissemination of one particular or different plasmids among these isolates.

140 **Antibiotic resistance profiles.** All 22 isolates exhibited phenotypes of ESBL producers
141 according to the CLSI scheme, showing inhibitable resistances to cefpodoxime (n=22),
142 cefotaxime (n=21) and ceftazidime (n=20). Beside this diverse resistance to cephalosporins,
143 the majority of isolates were resistant to aminoglycosides (n=19), fluoroquinolones (n=21),
144 tetracycline (n=17) and sulphonamides (n=22). After conjugation and selection on LB agar
145 containing cefotaxime, transfer of cefotaxime resistance could be observed in 17 cases.
146 Cotransfer of aminoglycoside (n=10), tetracycline (n=7) and sulphonamide resistance (n=3)
147 was observed. (Tab. 2)

148 **β-Lactamase gene distribution.** The most frequent β-lactamase genes found belonged
149 to $bla_{\text{CTX-M}}$ class (21/22), followed by $bla_{\text{TEM-1}}$ (14/22). Bla_{SHV} occurred only once,
150 accompanied by $bla_{\text{TEM-1}}$. While only one TEM type (TEM-1) was detected, six different
151 CTX-M types could be distinguished. Most of them were assigned to the CTX-M group 1 and
152 were classified as CTX-M-15 (12/22), CTX-M-1 (4/22) and CTX-M-3 (1/22). Several isolates
153 carried CTX-M group 9 genes as CTX-M-14 (2/22), CTX-M-9 (1/22) and the new variant
154 CTX-M-65 (1/22; GenBank acc. no. EF 418608) (Tab. 2).

155 **Molecular typing and phylogenetic grouping.** Half of the isolates (n=11) belonged to
156 phylogenetic group B2, seven to group D, three to group A, and one isolate was classified in
157 phylogroup B1 (Tab. 2). Eight different sequence types were determined of which the two
158 1574 and 1575 were newly assigned (Tab. 2). 27% (n=6) of the isolates were identified as the
159 internationally disseminated $bla_{\text{CTX-M-15}}$ containing *E. coli* clone O25:H4, ST131, phylogroup
160 B2 (Lau *et al.*, 2008). This rate has also been found in a three-year study of Blanco *et al.*
161 (2009). Interestingly, five isolates of phylogroup B2, ST131 exhibited other $bla_{\text{CTX-M}}$ types
162 ($bla_{\text{CTX-M-1}}$ (n=3), $bla_{\text{CTX-M-9}}$ (n=1) and $bla_{\text{CTX-M-65}}$ (n=1)). Up to now there are only two

isolates of phylogroup B2, ST131 reported exhibiting *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-3}}$, respectively (Blanco *et al.*, 2009; Woodford *et al.*, 2009), what confirmes the potential of plasmid hitchhiking by this epidemic strain predicted by Coque *et al.* (2008).

Among the isolates investigated 18 different PFGE patterns were discriminated (Tab. 2, Fig. 1). Only eight isolates exhibited patterns that allowed grouping in three distinct PFGE clusters (A, B, C). Therefore intrahospital spread of clones can widely be excluded except two unrelated cases, in which indistinguishable PFGE and similar plasmid and antibiotic resistance patterns (cluster B and C) were detected in isolates of patients hospitalized in the same time period and same wards. Strains belonging to PFGE cluster A were isolated from patients of different age, sex and hospitalized in different wards at different times, what suggests their introduction to the hospital from the community or acquisition during a previous stay in other hospitals. Altogether, the emergence of ESBL producing *E. coli* in the observed time period was mainly not associated with clonal dissemination of one particular strain as underlined by different PFGE, plasmid and antibiotic resistance patterns. This corresponds to earlier reports from other European countries as well as Canada (Mulvey *et al.*, 2004; Canton *et al.*, 2008). The polyclonal nature of the *E. coli* producing CTX-M β-lactamases in a nosocomial setting as described here could be explained by gut colonization and wide horizontal spread of *bla*$_{\text{CTX-M}}$ genes.

Plasmid analysis. All of the isolates exhibited different plasmid profiles, except those which shared indistinguishable PFGE clusters (Tab. 2, Fig. 1). *Bla*$_{\text{CTX-M}}$ containing plasmids of 17 isolates could be sole transferred by conjugation, showing sizes between 40 kbp and 180 kbp, estimated by means of plasmid size standards (Sherburne *et al.*, 2000; Macrina *et al.*, 1978). In five cases *bla*$_{\text{TEM-1}}$ were cotransferred. For all conjugative plasmids class 1 integron PCR was positive, but it were not associated with *bla*$_{\text{CTX-M-1}}$ or *bla*$_{\text{CTX-M-9}}$ as proven by long PCR. The most frequent CTX-M type *bla*$_{\text{CTX-M-15}}$ was most often located on plasmids belonging to incompatibility groups IncFII (n=7) and IncI1 (n=2). Other CTX-M-1 group

genes were located on IncN plasmids (n=4) and IncI1 plasmids (n=1). CTX-M-9 group genes were found on IncFII (n=2) and IncN (n=1) plasmids (Tab. 2). Cointegration of IncN, IncF and IncI like in virulence plasmid pCoo or multiple drug resistance plasmid pK245 (Chen *et al.*, 2006; Froehlich *et al.*, 2005) could be excluded, because respective incompatibility PCR resulted in demonstration of only one Inc determinant. For four isolates the conjugative transfer of the *bla*$_{\text{CTX-M}}$ carrying plasmid was not successful and consequently incompatibility group determination was not possible. This could either be due to localization of the gene at non-self-transmissible or rarely transferable plasmids or integration of *bla*$_{\text{CTX-M}}$ into the chromosome (Cao *et al.*, 2002; Chanawong *et al.*, 2002; Coque *et al.*, 2008). The demonstration of plasmids differing in size and incompatibility characteristics and the finding of the same *bla*$_{\text{CTX-M}}$ type in isolates harbouring obviously different plasmids indicate that there was no spread of one particular *bla*$_{\text{CTX-M}}$ containing plasmid among different *E. coli* strains. Recently published data for another German hospital showed widely unrelated ESBL producing *E. coli* strains with different plasmids, too (Mshana *et al.*, 2009).

Genetic environment of *bla*$_{\text{CTX-M}}$. This investigation should elucidate in which structure and where the *bla*$_{\text{CTX-M}}$ determinants integrate in different host plasmids. Therefore transconjugants were chosen for genetic environment analysis with regard to their diversity of CTX-M group 1 types within the same incompatibility groups and their relatedness according to PFGE profiles, respectively. From each incompatibility group at least two isolates were selected including both, clonally related and unrelated strains. In total nine isolates with CTX-M-1 (3xIncN, 1xIncI1), CTX-M-15 (3xIncFII, 1xIncI1) and CTX-M-65 (1xIncN) were analyzed.

Walking experiments identified the insertion sequence IS*Ecp1* upstream and in same orientation as the *bla*$_{\text{CTX-M}}$ gene in all selected isolates, but differing in sizes as well as their distances from *bla*$_{\text{CTX-M}}$ (Fig. 2). The upstream sequences for pKC394, pKC406, pKC409, pKCT 398 and pKC404 were identical to accession number FJ235692. The plasmids bearing

215 *bla*$_{\text{CTX-M-15}}$ carried 48 bp upstream of *bla*$_{\text{CTX-M-15}}$ the insertion sequences of IS*Ecp1* showing
216 different sizes. In detail pKC405 contained only the right IR of IS*Ecp1*, pKC390 contained a
217 387 bp IS*Ecp1* remnant and pKCT407 contained the whole IS element (identical to GenBank
218 accession number AY604721). All IS*Ecp1* elements, except for pKCT407, were disrupted by
219 an intact IS*26* located in opposite orientation. Downstream all CTX-M group 1 genes were
220 accompanied by a sequence similar to ORF477, truncated at nucleotide position 323 by an
221 IR-R of IS*Ecp1*. The genetic neighbourhood found in pKC396 (*bla*$_{\text{CTX-M-65}}$) was identical to
222 GenBank accession number AJ972953 and has been demonstrated for *bla*$_{\text{CTX-M-14}}$ (Eckert,
223 Gautier & Arlet, 2006). This implicates the generation of the new variant *bla*$_{\text{CTX-M-65}}$ in
224 pKC396 by two point mutations in *bla*$_{\text{CTX-M-14}}$.

225 *Bla*$_{\text{CTX-M}}$ genetic neighbourhood identical to pKC390, pKC396, pKC394, pKC406,
226 pKC409, pKCT398 and pKCT407, respectively, has already been described (Eckert *et al.*,
227 2006; Saladin *et al.*, 2002). But a solely inverted repeat of IS*Ecp1* 48 bp distant from the
228 *bla*$_{\text{CTX-M-15}}$ gene (in pKC405) has no more been reported before than a *bla*$_{\text{CTX-M-15}}$ gene
229 carrying 80 bp upstream a 214 bp IS*Ecp1* remnant (in pKC404), usually typical for *bla*$_{\text{CTX-M-1}}$.
230 Genetic rearrangement upstream of *bla*$_{\text{CTX-M-15}}$ concerning the IS*Ecp1* remnant must have
231 occurred during short time as the isolates 404 and 405 originated from different patients
232 sharing the same room.

233 There are only a few data on structures beyond the *bla*$_{\text{CTX-M}}$/IS*26* element (Literacka *et*
234 *al.*, 2009; Hall, 1987). The extended *bla*$_{\text{CTX-M}}$ genetic environment corresponding to that in
235 pKC394, 406, 409, 390 and pKCT398 is firstly described. Regarding the ORFs upstream and
236 downstream of the *bla*$_{\text{CTX-M}}$/IS*26* element (Fig. 2) it is conspicuously that the CTX-M/IS*26*
237 complex in IncN and IncI1 plasmids was surrounded by the same genes in clonally related
238 strains as well as in unrelated isolates. These were downstream entire *mphA* and partial *mrx*
239 genes, dedicated to an incomplete and therefore non-functional macrolide resistance gene
240 cluster. Furthermore, a second IS*26* copy, which showed a direct repeat (TTACCGGT)

corresponding to the IS*26* element upstream of *bla*$_{CTX-M}$ was detected. Genes found upstream of the CTX-M/IS*26* complex were NP_511181 encoding for a restriction endonuclease, Mrr_cat, flanked by NP_511180 and ORF2 coding for two hypothetical proteins of unknown functions. However, in the IncI1 plasmid pKC390 this environment was only partially detected and could not entirely be proven by confirmatory PCR. Although the genes NP_511180, NP_511181 and ORF2 (R46) were already previously described in IncN plasmids as well as *mrx* and *mphA* in IncF (pRSB101) and IncN (pLEW517) they were neither found to be that close together nor conjoint with *bla*$_{CTX-M}$ genes (Hall, 1987; Williams *et al.*, 2006; Szczepanowski *et al.*, 2004). Since there is a second IS*26* element orientated in the same direction, we suppose a novel IS*26* composite transposon in the plasmids pKC394, 406, 409 and pKCT398. *Bla*$_{ESBL}$ genes flanked by two IS*26* elements have been described before as part of composite transposons in different enterobacterial species (Garza-Ramos *et al.*, 2009; Doublet *et al.*, 2009). The finding of same gene arrangements in direct genetic neighbourhood of *bla*$_{CTX-M}$ in plasmids of incompatibility group IncN and IncI1 suggests the exchange of large *bla*$_{CTX-M}$ containing modules between different plasmid backbones. This was probably mediated by an IS*26* transposition event, which is indicated by two directly repeated IS*26* copies flanked by identical sequences 8 bp in size. Together with duplicated and same orientated IS elements this is typical for IS*26* transposition (Iida *et al.*, 1984). Same upstream sequences in plasmids of IncI1 as well as IncN could be explained by convergent integration of the *bla*$_{CTX-M}$-IS*26* composite transposon at the same sites in different plasmids. This is supported by two facts. Firstly, the direct repeats are identical in pKCT398 as well as in pKC394, 406 and 409. Secondly, in pKCT398 compared to IncN plasmids there are 42 additional nucleotides found between the *mphA* gene and the second IS*26* element. In other plasmids insertion sequence IS*26* was also found to be located in direct neighbourhood of *mphA* and NP511181, respectively, but at other nucleotide positions than found in pKC394, 406, 409 and pKCT398 (Hall, 1987; Szczepanowski *et al.*, 2004). Maybe sequence

similarities to IS*26* inverted repeats constitute a preferred IS*26* integration site in these genes. However, the idea of a large transposon like structure incorporated the *bla*$_{\text{CTX-M}}$-*IS26* element could not entirely be excluded. Lately, chromosomal integration of *bla*$_{\text{CTX-M-3a}}$ with two distantly located IS*26* elements has been demonstrated (Literacka *et al.*, 2009). Together with the IS*26* structure reported here, this points out the impressive changeability of IS*26* and underline the important role of IS*26* in spread of *bla*$_{\text{ESBL}}$ genes.

Accession numbers. The CTX-M surrounding sequences for strain 409, 390, 394, 396, 398, 404, 405, 406 and 407, respectively, are available at GenBank (www.ncbi.nlm.nih.gov) under accession numbers GQ274927 to GQ274935.

Acknowledgements

This work was funded by the German Ministry of Health (project ARS). We thank Dr G. A. Jacoby for giving the azide resistant *E. coli* strain and Dr A. Carattoli for providing Inc group reference strains.

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421 **Table 1: primers used in this study**; * in walking experiments only one primer was used

PCR experiment	primer 1	potential primer 2	sequence 5´→3´	target	T_a
ESBL multiplex	SHV fw		ttcgcctgtgtattatctcc	*bla*$_{SHV}$	55
	SHV rv		tccgctctgctttgttattc		55
	TEM fw		atgagtattcaacatttccg	*bla*$_{TEM}$	55
	TEM rv		ttaatcagtgaggcacctat		55
	CTX-M fw		cgctttgcgatgtgcag	group1	55
	CTX-M rv		accgcgatatcgttggt		55
	CTX-M 9 fw		gcagtacagcgacaataccg	*bla*$_{CTX-M}$ group 9	55
	CTX-M 9 rv		tatcattggtggtgccgtag		55
long PCR	L1_intI1_+91		cggttcgtaaactgtaatgcaagta	*bla*$_{CTX-M}$ integrase gene	67
	L2_sul1_+29		aaatccttggatatcgttcaggtagc	CR-fragment	67
	L3_sul1_+76		gaagcgcaatcaccttctcggaaa	CR-fragment	67
	L4a_X1cons_	L1-	cgtgaactggcgcagtgatttttt	*bla*$_{CTX-M-1}$ upstream	
	L4b_X9cons_	L1-	actgcacattggaaagcattcatca	*bla*$_{CTX-M-9}$ upstream	
	L5_intI1_+82		ccattccgacgtctctacgacgatg	integrase gene	70
	L7a_X1cons_	L1-	ggcagaaagccgtcgcgatgtatt	*bla*$_{CTX-M-1}$	
	L7b_X9cons_	L1-	cgccgctggttctggtgacctatttt	*bla*$_{CTX-M-9}$	
walking	TSP2_X1_+2	-*	cgctcatcagcacgataaag	*bla*$_{CTX-M-1}$ upstream	63
	TSP3_X1_+8	-	gcatacagcggcacacttc	*bla*$_{CTX-M-1}$ upstream	61
	TSP7_X1_+8	-	agccgtcgcgatgtattag	*bla*$_{CTX-M-1}$	56
	TSP8_IS26_+	-	ccaggcctcagcattttatt	IS*26* upstream	56
	TSP10	-	catcaccgcgataaagcacc	*bla*$_{CTX-M-65}$	56
	TSP13_X9_+	-	ggctcaaaggcaatacgacc	*bla*$_{CTX-M-65}$	56
confirmation	mphA_+614	TSP	atgtgctcatcgacaacac	*mphA*	61
PCR	ORF2_+196	TSP	tccagcggctattgctatct	*ORF2*	61
	ISEcp1_+75	TSP	taaagaccatgctctgcggt	IS*Ecp1*	61
	IS26_+10	TSP	caaagttagcgatgaggcag	IS*26*	61
	mrx_+762		gggctgttctcctcaatgat	*mrx*	55
	hypB_+679	mrx_	acgctagaaacgagcaccat	NP_811151	55
	IS26_+616	mrx_	caaagttagcgatgaggcag	IS*26*	55
	L5_fw		ttttaggtaacgcacgttgg	*Eco*RIIm	55
	S5a_rv	L5_f	ttgtcgttcaccacgaactc	*mphA*	55

Table 2: characteristics of clinical and conjugative strains[*]; * bold: characteristics of clinical isolates as well as of transconjugants; [†] determined by broth microdilution; [‡] incompatibility groups of conjugative plasmids; [¦] n.d. = not determined; [§] exhibits non-transferable SHV-5

no. of isolate	phylotype	ST	PFGEtype	plasmidpattern	Inc group [‡]		*bla* genes		antibiotic resistances [†]
							TEM type	CTX-M type	
384	D	648	A1	1a	n.d. [¦]		-	15	CTM, CTX, CAZ, KAN, NAL, CIP, CHL, OTE, SMZ, SXT
403	D	648	A2	1b	n.d.		-	15	CTM, CTX, CAZ, NAL, CIP, SMZ
404	B2	131	B1	2a	FII		-	15	**CTM, CTX, CAZ, KAN, STR**, AMK, NAL, CIP, **OTE, SMZ**, SXT
405	B2	131	B1	2b	FII		-	15	**CTM, CTX, CAZ, KAN, STR**, AMK, NAL, CIP, **OTE, SMZ**, SXT
406	B2	131	C1	3a		N	1	1	**CTM, CTX, CAZ**, NAL, CIP, SMZ, SXT
409	B2	131	C1	3b		N	1	1	**CTM, CTX, CAZ**, NAL, CIP, SMZ, SXT
394	B2	131	C1	3b		N	1	1	**CTM, CTX, CAZ**, NAL, CIP, CHL, SMZ, SXT
387	D	648	D	4	n.d.		1	15	CTM, CTX, CAZ, GEN, KAN, STR, NAL, CIP, CHL, OTE, SMZ, SXT
397	B2	131	E	5	n.d.		1	9	CTM, CTX, CAZ, NAL, CIP, SMZ
399	D	648	F	6	FII		-	15	**CTM, CTX, CAZ, GEN, KAN**, NAL, CIP, CHL, **OTE**, SMZ, SXT
402	B1	156	G	7	I1		**1**	-[§]	**CTM, CTX, CAZ, GEN, KAN**, AMK, **STR**, NAL, CIP, CHL, **OTE, SMZ**, SXT
398	A	398	H	8	I1		**1**	1	**CTM, CTX, CAZ, GEN, STR**, SMZ
400	D	1575	I	9	FII		1	14	**CTM, CTX, CAZ**, GEN, NAL, CIP, CHL, OTE, SMZ, SXT
393	D	405	J	10	FII		-	15	**CTM, CTX, CAZ**, KAN, STR, NAL, CIP, **OTE**, SMZ
386	A	88	K	11	FII		**1**	14	**CTM, CTX, CAZ**, KAN, STR, OTE, SMZ, SXT
395	A	1574	L	12		N	**1**	3	**CTM, CTX, CAZ**, KAN, STR, NAL, CIP, CHL, OTE, **SMZ, SXT**
390	D	405	M	13	I1		1	15	**CTM, CTX, CAZ**, GEN, SMZ
392	B2	131	N	14	FII		-	15	**CTM, CTX, CAZ, GEN**, NAL, CIP, **OTE**, SMZ
407	B2	131	O	15	FII		**1**	15	**CTM, CTX, CAZ, KAN**, NAL, CIP, **OTE**, SMZ
396	B2	131	P	16		N	1	65	**CTM, CTX, CAZ**, GEN, STR, NAL, CIP, OTE, SMZ, SXT
385	B2	131	Q	17	I1		-	15	**CTM, CTX, CAZ, GEN, KAN**, NAL, CIP, CHL, **OTE**, SMZ
388	B2	131	R	18	FII		1	15	**CTM, CTX, CAZ, GEN, KAN**, STR, NAL, CIP, CHL, OTE, SMZ, SXT

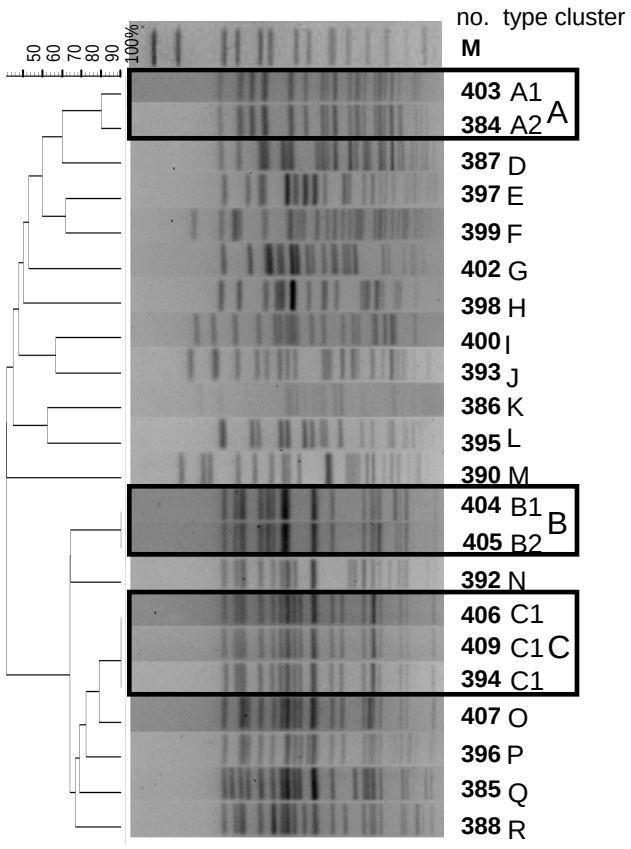

Figure 1: PFGE based dendrogramm of 22 ESBL producing *E.coli* isolates,
framed: strains with similarity ≥ 90 %; M= molecular size marker, PFGE standard: *Salmonella* serovar Breanderup

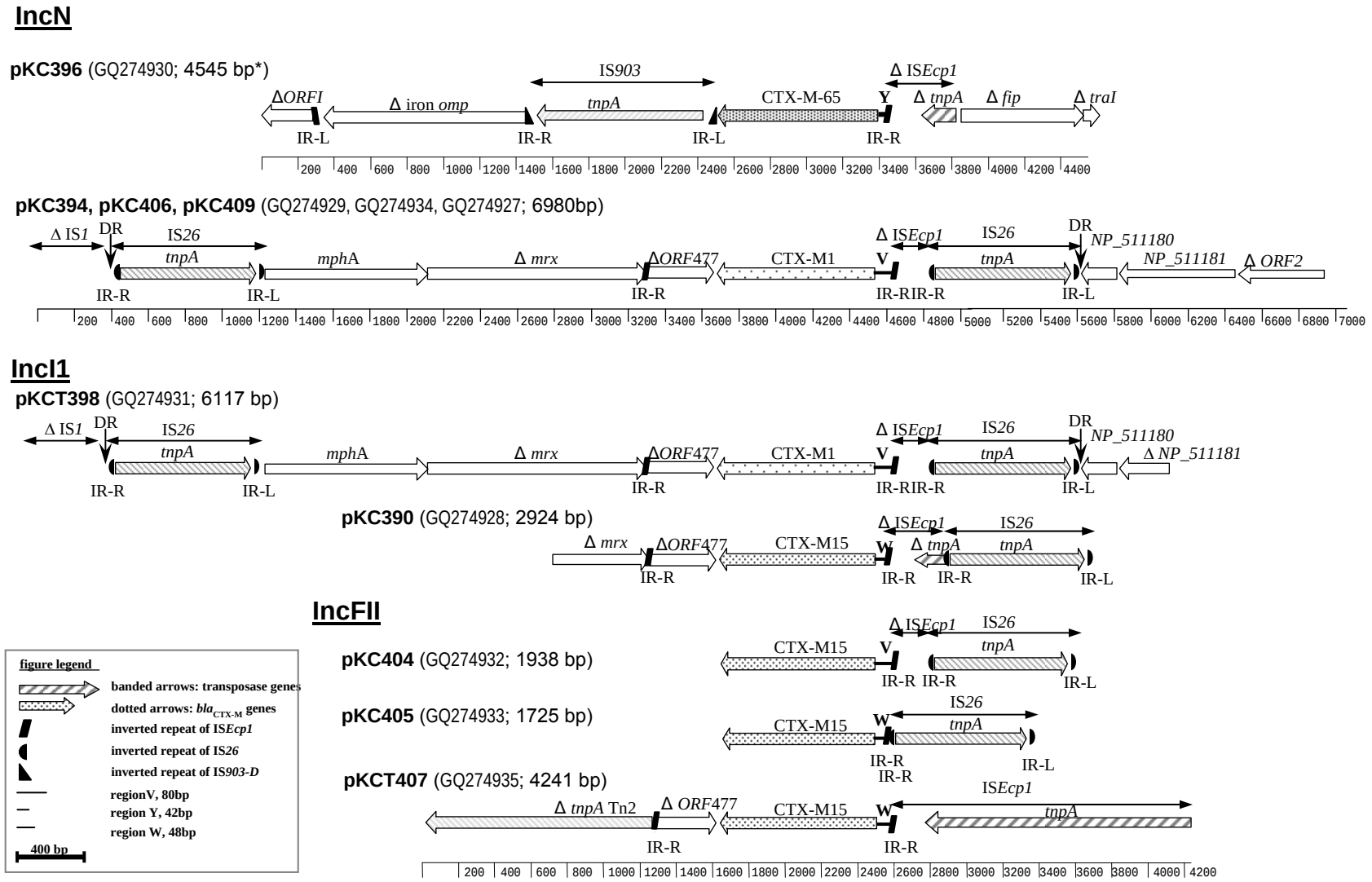

Figure 2: genetic maps of CTX-M environment; * sequence lengths explored by walking experiments; arrows: open reading frames, banded arrows: transposase genes, dotted arrows: $bla_{\text{CTX-M}}$ genes, white arrows: other neighbouring genes, filled symbols: inverted repeats specific to each IS; regions V, Y, W according to Eckert *et al*. (2006), DR: direct repeat of IS*26* (TTACCGGT)