A novel IS26 structure surrounds bla_{CTX-M} genes in different plasmids from German clinical Escherichia coli isolates

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This report focuses on the molecular characterization of 22 extended-spectrum β-lactamase-producing Escherichia coli isolates collected in a German university hospital during a period of 9 months in 2006. Relationship analysis of clinical isolates was done via PFGE, multilocus sequence typing, plasmid profiling and additionally PCR for bla_{ESBL} detection and determination of phylogroups. After conjugal transfer, plasmid isolation and subsequent PCR for bla_{ESBL} detection and determination of incompatibility groups were performed. Using one-primer walking, up to 3600 bp upstream and downstream of different bla_{CTX-M} genes could be sequenced. β-Lactamases found were TEM-1 (n = 14), SHV-5 (n = 1) and a wide variety of CTX-M types (n = 21), i.e. 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) and one new type, CTX-M-65 (n = 1). In 18 isolates, bla_{ESBL} genes were located on conjugative plasmids of sizes between 40 and 180 kbp belonging to incompatibility groups FII (n = 9), N (n = 5) and I1 (n = 4). bla_{CTX-M} was found to be associated with the common elements IS\text{Ecp1}, IS26 and IS903-D, but with unusual spacer sequences for IS\text{Ecp1} in two isolates. These insertion sequences, connected to bla_{CTX-M} as well as other genes, were located between two IS26 elements in a configuration that has not yet been described. The results reveal the emergence of bla_{ESBL}, predominantly bla_{CTX-M}, located on different plasmids harboured by genotypically different E. coli strains. The identical gene arrangement in the bla_{CTX-M} neighbourhood in plasmids of different incompatibility groups indicates a main role of IS26 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 (ESBLs) such as bla_{TEM}, bla_{SHV} and bla_{CTX-M} (Paterson & Bonomo, 2005). CTX-M-type β-lactamases seemed to be particularly successful in terms of spread. Since the first description in 1989, 86 variants have been found 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). Chromosomal genes of different Kluyvera species have been identified as a natural reservoir. A 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 acquired by different events (Rodriguez et al., 2004). A number of genetic mechanisms have apparently been involved in acquisition of CTX-M genes. Insertion sequences IS26, IS\text{Ecp1} and IS\text{CR1} 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\text{Ecp1} elements and remnants constitute an alternative promoter region (Karim et al., 2001) leading to increased, clinically relevant expression of the bla_{CTX-M} gene, which 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 from 40 to over 200 kb (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; Dietstra 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 *Escherichia coli* strains in a university hospital. We elucidate the blaCTX-M environment in selected isolates concerning different CTX-M types on plasmids of different incompatibility groups. Analysis of the genetic environment of blaCTX-M genes can reveal details of acquisition with regard to their origin and further dissemination.

**METHODS**

**Bacterial strains.** During a period of 9 months from the end of January to the beginning of October in 2006, 22 *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 hospitalized in urology (35 %), surgery (27 %) as well as several other (38 %) wards. Patients were between 3 weeks and 87 years old (51 years on average); 50 % were male and 50 % were female. Eleven patients had been treated with fluoroquinolones and/or β-lactam antibiotics previously.

**Antimicrobial susceptibility testing.** Standard microbroth dilution assay, according to a CLSI protocol, was performed and resistance to 17 commonly used antibiotics belonging to different antibiotic classes was assessed (ampicillin, mezlocillin, mezlocillin–sulbactam, cefotiam, cefotaxime, ceftazidime, cefoxitin, gentamicin, kanamycin, amikacin, streptomycin, nalidixic acid, chloramphenicol, oxytetracycline, ciprofloxacin, sulfametoxazol and sulfametoxazol–trimethoprim) (NCCLS, 2000). Phenotypic identification of ESBL producers was performed in a second, confirmatory microbroth dilution test detecting the resistance to three third-generation cephalosporins (cefotaxime, ceftazidime and cefpodoxime) in the presence and absence of clavulanic acid (NCCLS, 1997, 1999).

**Clonal characterization of *E. coli* isolates.** PFGE was performed following the protocol of Hunter et al. (2005). TIFF files were analysed using BioNumerics software. Similarity values were computed using the Dice coefficient and visualized in a dendrogram based on the UPGMA method. Strains showing >90 % similarity were classified as genetically related and assigned to the same lineage. All isolates were further analysed by multilocus sequence typing (MLST) following the official protocol of the MLST database (http://mlst.ucc.ie/mlst/db/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 PCRs in this study were done using Taq polymerase (Invitrogen) and run on an ABI capillary sequencer. The nucleotide sequences were analysed with Lasergene software and compared with data submitted to the NCBI sequence database using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/blast/).

**Plasmid analysis.** Transfer of blaCTX-M-1 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 (1996).

Plasmid DNA of donor and transconjugants was isolated using the Plasmid Mini kit (Qiagen) and analysed on 0.4 % agarose gels using *E. coli* V517 and *E. coli* R27 as size markers (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 was performed as previously described by Mazel et al. (2000) and Carattoli et al. (2005), respectively, using DNA from plasmid mini preparations of transconjugants and whole genomic DNA of the recipient strain as a negative control.

**Genetic environment of blaCTX-M.** Integron association of blaCTX-M genes was determined by long PCR using the DyNAzyme EXT PCR kit (Finnzymes) according to the manufacturer’s instructions. Primers are listed in Table 1.

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

Confirmation of newly explored sequences accompanying blaCTX-M genes was performed by PCR using primers and annealing temperatures listed in Table 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 (Amersham Biosciences). The isolated fragments were subsequently ligated into a pCR 2.1 vector and transformed into chemically competent *E. coli* K-12 TOP10F′ using the TA Cloning kit (Invitrogen) according to the manufacturer’s instructions. Plasmid inserts were amplified using M13 primers. When showing the expected sizes, inserts were sequenced and analysed as described above.

**RESULTS AND DISCUSSION**

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

**Antibiotic resistance profiles**

All 22 isolates exhibited phenotypes of ESBL producers according to the NCCLS (2000) scheme, showing inhibitable resistance to cephalosporins (n=22), cefotaxime (n=21) and ceftazidime (n=20). Beside this diverse resistance to cephalosporins, the majority of isolates were
The primers used in this study are listed in Table 1. After conjugation and selection on LB agar containing tetracycline ($n=5$), cotransfer of aminoglycoside ($n=21$), fluoroquinolones ($n=17$) and sulphonamides ($n=22$). After conjugation and selection on LB agar containing cefotaxime, transfer of cefotaxime resistance could be observed in 18 cases. Cotransfer of aminoglycoside ($n=9$), tetracycline ($n=7$) and sulphonamide ($n=4$) resistance was observed (Table 2).

### β-Lactamase gene distribution

The most frequent β-lactamase genes found belonged to the bla$_{\text{CTX-M}}$ class (21/22), followed by bla$_{\text{TEM-1}}$ (14/22). bla$_{\text{SHV}}$ occurred only once, accompanied by bla$_{\text{TEM-1}}$. While only one TEM type (TEM-1) was detected, six different CTX-M types could be distinguished. Most of them were assigned to CTX-M group 1 and were classified as CTX-M-15 (12/22), CTX-M-1 (4/22) and CTX-M-3 (1/22). Several isolates carried CTX-M group 9 genes CTX-M-14 (2/22), CTX-M-9 (1/22) and the new variant, CTX-M-65 (1/22; GenBank accession no. EF418608) (Table 2).

### Molecular typing and phylogenetic grouping

Half of the isolates ($n=11$) belonged to phylogroup B2, seven belonged to group D, three belonged to group A and one isolate was classified in phylogroup B1 (Table 2). Eight different sequence types were determined of which two, 1574 and 1575, were newly assigned (Table 2). Twenty-seven percent ($n=6$) of the isolates were identified as the internationally disseminated bla$_{\text{CTX-M-15}}$-containing _E. coli_ clone O25:H4, ST131, phylogroup B2 (Lau et al., 2008). This rate was also found in the 3-year study of Blanco et al. (2009). Interestingly, five isolates of phylogroup B2, ST131, exhibited other bla$_{\text{CTX-M}}$ types [bla$_{\text{CTX-M-1}}$ (n=1), bla$_{\text{CTX-M-15}}$ (n=1) and bla$_{\text{CTX-M-65}}$ (n=1)]. Up to now, only two isolates of phylogroup B2, ST131, have been reported exhibiting bla$_{\text{CTX-M-14}}$ and bla$_{\text{CTX-M-30}}$ respectively (Blanco et al., 2009; Woodford et al., 2009), which confirms 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 (Table 2, Fig. 1). Only eight isolates...
Table 2. Characteristics of clinical and conjugative strains

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ND, not determined.
*Incompatibility groups of conjugative plasmids.
†Determined by broth microdilution. CTM, cefotiam; CTX, cefotaxime; CAZ, ceftazidime; KAN, kanamycin; NAL, nalidixic acid; CIP, ciprofloxacin; CHL, chloramphenicol; OTE, oxytetracycline; SMZ, sulfameracin; SXT, sulfameracin–trimethoprim; STR, streptomycin; AMK, amikacin; GEN, gentamicin.
§Exhibits non-transferable SHV-5.
Plasmid analysis

All of the isolates exhibited different plasmid profiles, except those which shared indistinguishable PFGE clusters (Table 2, Fig. 1). blaCTX-M-containing plasmids of 17 isolates could be solely transferred by conjugation and showed 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, blaTEM-1 was cotransferred. For all conjugative plasmids, class 1 integron PCR was positive, but it was not associated with blaCTX-M-1 or blaCTX-M-9 as proven by long PCR. The most-frequent CTX-M type, blaCTX-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 found on IncN (n=4) and IncI1 (n=1) plasmids. CTX-M-9 group genes were found on IncFII (n=2) and IncN (n=1) plasmids (Table 2). Cointegration of IncN, IncF and IncI as 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 blaCTX-M-carrying plasmid was not successful and consequently incompatibility group determination was not possible. This could be due to either localization of the gene at non-self-transmissible or rarely transferable plasmids or integration of blaCTX-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 blaCTX-M type in isolates harbouring obviously different plasmids indicate that there was no spread of one particular blaCTX-M-containing plasmid among different E. coli strains. Recently published data for another German hospital also showed widely unrelated ESBL-producing E. coli strains with different plasmids (Mshana et al., 2009).

Genetic environment of blaCTX-M

This investigation should elucidate in which structure and where the blaCTX-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. 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 (3 x IncN, 1 x IncI1), CTX-M-15 (3 x IncFII, 1 x IncI1) and CTX-M-65 (1 x IncN) were analysed.

Walking experiments identified the insertion sequence ISecp1 upstream and in the same orientation as the blaCTX-M gene in all selected isolates, but differing in size as well as the distance from blaCTX-M (Fig. 2). The upstream sequences for pKC394, pKC406, pKC409, pKCT398 and pKC404 were identical to the sequence with accession number FJ235692. The plasmids bearing blaCTX-M-15 carried 48 bp upstream of blaCTX-M-15 the insertion sequences of ISecp1 showing different sizes. In detail, pKC405 contained only the right IR of ISecp1, pKC390 contained a 387 bp ISecp1 remnant and pKC407 contained the whole IS element (identical to the sequence with GenBank accession no. AY604721). All ISecp1 elements, except for pKC407, were disrupted by an intact IS26 located in the opposite orientation. Downstream all CTX-M group 1 genes were accompanied by a sequence similar to that of ORF477, truncated at nucleotide position 323 by an IR-R of ISecp1. The genetic neighbourhood found in pKC396 (blaCTX-M-65) was identical to the sequence with GenBank accession number AJ972953 and has been demonstrated for blaCTX-M-1 (Eckert et al., 2006). This implies that the new variant blaCTX-M-65 in pKC396 was generated by two point mutations in blaCTX-M-14.

A blaCTX-M genetic neighbourhood identical to pKC390, pKC396, pKC406, pKC409, pKCT398 and pKCT407 has already been described (Eckert et al., 2006; Saladin et al., 2002). However, a solely inverted repeat of ISecp1 48 bp from the blaCTX-M-15 gene (in pKC405) has not been reported before nor has a blaCTX-M-15 gene carrying a 214 bp ISecp1 remnant 80 bp upstream (in pKC404), usually typical for blaCTX-M-1. Genetic rearrangement
upstream of bla$_{CTX-M-15}$ concerning the IS$Ecp1$ remnant must have occurred over a short time as isolates 404 and 405 originated from different patients sharing the same room.

There are only a few data on structures beyond the bla$_{CTX-M}$/IS$26$ element (Literacka et al., 2009; Hall, 1987). The extended bla$_{CTX-M}$ genetic environment corresponding to that in pKC394, pKC406, pKC409 and pKCT398 is firstly described. Regarding the ORFs upstream and downstream of the bla$_{CTX-M}$/IS$26$ element (Fig. 2), it is clear that the CTX-M/IS$26$ complex in IncN and IncI1 plasmids is surrounded by the same genes in clonally related strains as in unrelated isolates. These were downstream entire mphA and partial mrxA genes, dedicated to an incomplete and therefore non-functional macrolide resistance gene 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 a restriction endonuclease, Mrr$cat$, flanked by NP$_{511180}$, and ORF2, encoding 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) have already been previously described in IncN plasmids as well as mrxA and mphA in IncF (pRSB101) and IncN (pLEW517), they were not found to be that close together or 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 propose that there is a novel IS$26$ composite transposon in plasmids pKC394, pKC406, pKC409 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 the same gene arrangements in the direct genetic neighbourhood of bla$_{CTX-M}$ in plasmids of incompatibility groups 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 IS

Fig. 2. Genetic maps of the CTX-M environment. *Sequence lengths explored by walking experiments; arrows, open reading frames; banded arrows, transposase genes; dotted arrows, bla$_{CTX-M}$ 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).
elements that are duplicated and in the same orientation this is typical for IS26 transposition (Iida et al., 1984). The same upstream sequences in plasmids of IncI1 as well as IncN could be explained by convergent integration of the blaCTX-M/IS26 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, pKC406 and pKC409. Secondly, there are 42 additional nucleotides found between the mphA gene and the second IS26 element in pKCT398 compared to IncN plasmids. In other plasmids, IS26 was also found to be located in the direct neighbourhood of mphA and NP_511181, but at nucleotide positions other than found in pKC394, pKC406, pKC409 and pKCT398 (Hall, 1987; Szczepanski et al., 2004). Maybe sequences similar to IS26 inverted repeats constitute a preferred IS26 integration site in these genes.

However, the idea of a large transposon-like structure incorporating the blaCTX-M/IS26 element could not entirely be excluded. Recently, chromosomal integration of blaCTX-M-3a with two distantly located IS26 elements has been demonstrated (Literacka et al., 2009). Together with the IS26 structure reported here, this highlights the impressive changeability of IS26 and underlines the important role of IS26 in spread of blaESBL genes.

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