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 *ISEcp1*, IS26 and IS903-D, but with unusual spacer sequences for *ISEcp1* 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.

Received 25 September 2009

Accepted 20 January 2010

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 (Tzouveleakis *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, *ISEcp1* and *ISCR1* 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, *ISEcp1* 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; Diestra *et al.*, 2009). A large number of them are conjugative, facilitating intra- and interspecies spread. Here

Abbreviation: ESBL, extended-spectrum β -lactamase.

The GenBank/EMBL/DDBJ accession number for the newly determined sequence of *bla*_{CTX-M-65} is EF418608, and those for the *bla*_{CTX-M} surrounding sequences in isolates 409, 390, 394, 396, 398, 404, 405, 406 and 407 are GQ274927–GQ274935, respectively.

we report pheno- and genotypic analyses of a collection of ESBL-producing *Escherichia 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. Analysis of the genetic environment of *bla*_{CTX-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, sulfameracin and sulfameracin-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/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 PCRs in this study were done using illustra PuReTaq Ready-To-Go beads (Amersham Biosciences) according to the manufacturer's instructions.

ESBL identification. The ESBL resistance genes (*bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}) were amplified by multiplex PCR and subsequently sequenced using previously described primers (Grobner *et al.*, 2009). 0.4 μ l CTX-M primers, 0.4 μ l SHV primers, 0.3 μ l CTX-M-9 primers and 0.9 μ l TEM primers (concn=10 pmol μ l⁻¹) were used. After an initial denaturation at 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 a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit 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 *bla*_{CTX-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 (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 *bla*_{CTX-M}. Integron association of *bla*_{CTX-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 *bla*_{CTX-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 *bla*_{CTX-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 cefpodoxime ($n=22$), cefotaxime ($n=21$) and ceftazidime ($n=20$). Beside this diverse resistance to cephalosporins, the majority of isolates were

Table 1. Primers used in this study

PCR experiment	Primer 1	Potential primer 2	Sequence (5'→3')	Target	T _a °C
ESBL multiplex	SHV fw		TTCGCCTGTGTATTATCTCC	<i>bla</i> _{SHV}	55
	SHV rv		TCCGCTCTGCTTTGTATTTC		55
	TEM fw		ATGAGTATTCAACATTTCCG	<i>bla</i> _{TEM}	55
	TEM rv		TTAATCAGTGAGGCACCTAT		55
	CTX-M fw		CGCTTTGCGATGTGCAG	Group 1 <i>bla</i> _{CTX-M}	55
	CTX-M rv		ACCGCGATATCGTTGGT		55
	CTX-M 9 fw		GCAGTACAGCGACAATACCG	Group 9 <i>bla</i> _{CTX-M}	55
	CTX-M 9 rv		TATCATTGGTGGTGCCGTAG		55
					55
Long PCR	L1_intl1_+914		CGGTTTCGTAACCTGTAATGCAAGTAGCGTAT	Integrase gene	67
	L2_sul1_+298		AAATCCTTGGATATCGTTTCAGGTAGCCCA	ISCR1 fragment	67
	L3_sul1_+760		GAAGCGCAATCACCTTCTCGGAAAC	ISCR1 fragment	67
	L4a_X1cons_+6	L1-3;L5	CGTGAACCTGGCGCAGTGATTTTTT	<i>bla</i> _{CTX-M-1} upstream	
	L4b_X9cons_+192	L1-3;L5	ACTGCACATTGGAAAGCATTTCATCACC	<i>bla</i> _{CTX-M-9} upstream	
	L5_intl1_+82		CCATTCCGACGTCTCTACGACGATGAT	Integrase gene	70
	L7a_X1cons_+815	L1-3;L5	GGCAGAAAGCCGTCGCGATGTATTAG	<i>bla</i> _{CTX-M-1} downstream	
	L7b_X9cons_+776	L1-3;L5	CGCCGCTGGTTCGTTGACCTATTTTAC	<i>bla</i> _{CTX-M-9} downstream	
Walking*	TSP2_X1_+203	—	CGCTCATCAGCACGATAAAAG	<i>bla</i> _{CTX-M-1} upstream	63
	TSP3_X1_+83	—	GCATACAGCGGCACACTTC	<i>bla</i> _{CTX-M-1} upstream	61
	TSP7_X1_+821	—	AGCCGTCGCGATGTATTAG	<i>bla</i> _{CTX-M-1} downstream	56
	TSP8_IS26_+696	—	CCAGGCCTCAGCATTTTATT	IS26 upstream	56
	TSP10_X9_+199	—	CATCACCGCGATAAAGCACC	<i>bla</i> _{CTX-M-65} upstream	56
	TSP13_X9_+638	—	GGCTCAAAGGCAATACGACC	<i>bla</i> _{CTX-M-65} downstream	56
Confirmation PCR	mphA_+614	TSP7; ORF2	ATGTGCTCATCGACAACAC	<i>mphA</i>	61
	ORF2_+196	TSP3	TCCAGCGGCTATTGCTATCT	ORF2	61
	ISEcp1_+75	TSP3	TAAAGACCATGCTCTGCGGT	ISEcp1	61
	IS26_+10	TSP3	CAAAGTTAGCGATGAGGCAG	IS26	61
	mrx_+762		GGGCTGTTCTCCTCAATGAT	<i>mrx</i>	55
	hypB_+679	mrx_+762	ACGCTAGAAACGAGCACCAT	NP_811151	55
	IS26_+616	mrx_+762	CAAAGTTAGCGATGAGGCAG	IS26	55
	L5_fw		TTTtaggTAACGCACGTTGG	<i>EcoRI</i> m	55
	S5a_rv	L5_fw	TTGTCGTTACACACGAACTC	<i>mphA</i>	55

*In walking experiments, only one primer was used.

resistant to aminoglycosides ($n=19$), fluoroquinolones ($n=21$), tetracycline ($n=17$) 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*_{CTX-M} class (21/22), followed by *bla*_{TEM-1} (14/22). *bla*_{SHV} occurred only once, accompanied by *bla*_{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 phylogenetic group 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 per cent ($n=6$) of the isolates were identified as the internationally disseminated *bla*_{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*_{CTX-M} types [*bla*_{CTX-M-1} ($n=3$), *bla*_{CTX-M-9} ($n=1$) and *bla*_{CTX-M-65} ($n=1$)]. Up to now, only two isolates of phylogroup B2, ST131, have been reported exhibiting *bla*_{CTX-M-14} and *bla*_{CTX-M-3}, 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

Bold indicates characteristics of clinical isolates as well as of transconjugants.

No. of isolate	Phylotype	ST	PFGE type	Plasmid pattern	Inc group*	bla genes		Antibiotic resistances†
						TEM type	CTX-M type	
384	D	648	A1	1a	ND	—	15	CTM, CTX, CAZ, KAN, NAL, CIP, CHL, OTE, SMZ, SXT
403	D	648	A2	1b	ND	—	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	ND	1	15	CTM, CTX, CAZ, GEN, KAN, STR, NAL, CIP, CHL, OTE, SMZ, SXT
397	B2	131	E	5	ND	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	II	1	—§	CTM, CTX, CAZ, GEN, KAN, AMK, STR , NAL, CIP, CHL, OTE, SMZ , SXT
398	A	398	H	8	II	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	II	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	II	—	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

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.

exhibited patterns that allowed grouping in three distinct PFGE clusters (A, B, C). Therefore intrahospital spread of clones can widely be excluded, except for two unrelated cases, in which indistinguishable PFGE and similar plasmid and antibiotic resistance patterns (cluster B and C) were detected in isolates from patients hospitalized in the same time period and same wards. Strains belonging to PFGE cluster A were isolated from patients of different age and sex and hospitalized in different wards at different times, which 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 not mainly 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*_{CTX-M} genes.

Fig. 1. PFGE-based dendrogram of 22 ESBL-producing *E. coli* isolates. Strains with similarity $\geq 90\%$ are boxed. M, Molecular size marker; PFGE standard, *Salmonella* serovar Braenderup.

Plasmid analysis

All of the isolates exhibited different plasmid profiles, except those which shared indistinguishable PFGE clusters (Table 2, Fig. 1). *bla*_{CTX-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, *bla*_{TEM-1} was cotransferred. For all conjugative plasmids, class 1 integron PCR was positive, but it was not associated with *bla*_{CTX-M-1} or *bla*_{CTX-M-9} as proven by long PCR. The most-frequent CTX-M type, *bla*_{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 ($n=4$) 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 *bla*_{CTX-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 *bla*_{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*_{CTX-M} type in isolates harbouring obviously different plasmids indicate that there was no spread of one particular *bla*_{CTX-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 *bla*_{CTX-M}

This investigation should elucidate in which structure and where the *bla*_{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. 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 ($3 \times$ IncN, $1 \times$ IncI1), CTX-M-15 ($3 \times$ IncFII, $1 \times$ IncI1) and CTX-M-65 ($1 \times$ IncN) were analysed.

Walking experiments identified the insertion sequence *ISEcp1* upstream and in the same orientation as the *bla*_{CTX-M} gene in all selected isolates, but differing in size as well as the distance from *bla*_{CTX-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 *bla*_{CTX-M-15} carried 48 bp upstream of *bla*_{CTX-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 pKCT407 contained the whole IS element (identical to the sequence with GenBank accession no. AY604721). All *ISEcp1* elements, except for pKCT407, 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 (*bla*_{CTX-M-65}) was identical to the sequence with GenBank accession number AJ972953 and has been demonstrated for *bla*_{CTX-M-14} (Eckert *et al.*, 2006). This implies that the new variant *bla*_{CTX-M-65} in pKC396 was generated by two point mutations in *bla*_{CTX-M-14}.

A *bla*_{CTX-M} genetic neighbourhood identical to pKC390, pKC396, pKC394, 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 *bla*_{CTX-M-15} gene (in pKC405) has not been reported before nor has a *bla*_{CTX-M-15} gene carrying a 214 bp *ISEcp1* remnant 80 bp upstream (in pKC404), usually typical for *bla*_{CTX-M-1}. Genetic rearrangement

IncN**pKC396** (GQ274930; 4545 bp*) **pKC394, pKC406, pKC409** (GQ274929, GQ274934, GQ274927; 6980bp) **IncI1****pKCT398** (GQ274931; 6117 bp) **pKC390** (GQ274928; 2924 bp) **IncFII****pKC404** (GQ274932; 1938 bp) **pKC405** (GQ274933; 1725 bp) **pKCT407** (GQ274935; 4241 bp)

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 IS26 (TTACCGGT).

upstream of *bla*_{CTX-M-15} concerning the *ISEcp1* 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}/IS26 element (Literacka *et al.*, 2009; Hall, 1987). The extended *bla*_{CTX-M} genetic environment corresponding to that in pKC394, pKC406, pKC409, pKC390 and pKCT398 is firstly described. Regarding the ORFs upstream and downstream of the *bla*_{CTX-M}/IS26 element (Fig. 2), it is clear that the CTX-M/IS26 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 *mrx* genes, dedicated to an incomplete and therefore non-functional macrolide resistance gene cluster. Furthermore, a second IS26 copy, which showed a direct repeat (TTACCGGT) corresponding to the IS26 element upstream of *bla*_{CTX-M}, was detected. Genes found upstream of the CTX-M/IS26 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 *mrx* 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 IS26 element orientated in the same direction, we propose that there is a novel IS26 composite transposon in plasmids pKC394, pKC406, pKC409 and pKCT398. *bla*_{ESBL} genes flanked by two IS26 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 IS26 transposition event, which is indicated by two directly repeated IS26 copies flanked by identical sequences 8 bp in size. Together with IS

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 *bla*_{CTX-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; Szczepanowski *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 *bla*_{CTX-M}/IS26 element could not entirely be excluded. Recently, chromosomal integration of *bla*_{CTX-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 *bla*_{ESBL} genes.

ACKNOWLEDGEMENTS

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

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