Molecular characterization of plasmids encoding CTX-M-15 β-lactamases from Escherichia coli strains in the United Kingdom

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Received 2 May 2006; returned 14 June 2006; revised 30 June 2006; accepted 7 July 2006

Objectives: The UK, like other countries worldwide, has a growing problem with CTX-M β-lactamase-producing Escherichia coli. Five major clonally related strains have been identified among CTX-M-15 producers. We characterize here the plasmids from clonal strains A and D.

Methods: Plasmids were extracted and transformed into E. coli DH5α; conjugative mating was attempted on agar. MICs were determined by agar dilution. β-Lactamases were typed by isoelectric focusing; antibiotic resistance genes and integrons were identified by PCR and sequenced. Plasmid incompatibility groups were determined by replicon PCR.

Results: blaCTX-M-15 was carried by a 150 kb plasmid in strain A and a 70 kb plasmid in strain D. Conjugative transfer of cefotaxime resistance was only achieved from strain D; plasmids from both strains were transferred by transformation. The plasmid from strain A additionally carried blaTEM-1 (variably), blaOXA-1, aac(6')-ib-cr and tet(A), as well as a class 1 integron with the gene cassettes aadA5 and dfr17; the plasmid from strain D carried blaTEM-1 consistently, also blaOXA-1, aac(6')-ib-cr, aac3-lla and tet(A). Both plasmids belonged to incompatibility group FII.

Conclusions: blaCTX-M-15 was plasmid-mediated in both strains A and D and was linked to other antibiotic resistance genes including aac(6')-ib-cr, which encodes an acetyltransferase, not previously found in Europe, acting on both aminoglycosides and some fluoroquinolones. Although the plasmids from the two strains differed in size, both were related and confered similar multi-drug resistance phenotypes, suggesting that they may share a similar genetic scaffold. Both shared features with plasmids encoding CTX-M-15 β-lactamases in E. coli from Canada and India.

Keywords: ESBLs, multi-drug resistance, aminoglycoside acetyltransferase

Introduction

CTX-M extended-spectrum β-lactamases (ESBLs) were first described in 1986¹ and have been a problem in South America since the early 1990s. Since around 2000, their prevalence and diversity have increased worldwide, with more than 50 variants now described, belonging to five evolutionary groups (http://www.lahey.org/studies/values.htm). CTX-M-15 is among the commonest CTX-M variants, having been described in many genera of Enterobacteriaceae across the world, and being the most-reported variant in much of Europe, except the Iberian peninsula.¹ Unusually among CTX-M enzymes, CTX-M-15 hydrolyses ceftazidime readily.²

Enterobacteriaceae with CTX-M enzymes have been associated with both hospital-acquired and community infections, mostly of the urinary tract.³⁴ Since 2003, the Antibiotic Resistance Monitoring and Reference Laboratory has identified over 1500 CTX-M producers, mostly Escherichia coli with CTX-M-15 enzymes, from over 150 centres in the UK.⁴⁵ The epidemiology of these E. coli isolates is partly clonal and includes five major strains (A–E, all serotyped as O25) as well as many diverse producers.⁴ Strain A, which is nationally widespread including in Shropshire, and strain D, which has only been reported in Shropshire,⁶ are the most closely distinct of the major lineages based on PFGE, and the present study sought to characterize the plasmids responsible for their multi-drug resistance.

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Materials and methods

Bacterial isolates

A total of 14 E. coli isolates with CTX-M-15 β-lactamase were investigated, comprising 11 strain A representatives from different UK centres and 3 strain D isolates from a single UK centre. Strain types were defined by PFGE of XbaI-digested genomic DNA.4 E. coli J53-2 (NCTC 50006; pro mer Rif) was the recipient strain for conjugation experiments, and E. coli DH5α the recipient for transformation.

Plasmid extraction, transfer and incompatibility typing

Plasmids were extracted by alkaline lysis and transformed into E. coli DH5α by electroporation at 2.5 kV, 25 μF and 200 Ω using a Gene-Pulser (Bio-Rad, Hemel Hempstead, UK). Transformants were selected on nutrient agar containing 100 mg/L rifampicin (Sigma-Aldrich, Poole, UK). Conjugal transfer of cefotaxime resistance to E. coli J53-2 was performed on nutrient agar plates with cultures grown into logarithmic phase. Transconjugants were selected on nutrient agar supplemented with 100 mg/L rifampicin (Sigma-Aldrich) and 2 mg/L cefotaxime. Plasmid incompatibility grouping was performed using transformants by a PCR-based replicon typing method.8

Antibiotic susceptibility testing

MICs were determined by Etest (AB BIODISK, Solna, Sweden) and agar dilution and were interpreted according to BSAC guidelines.9

Isoelectric focusing (IEF)

β-Lactamases were extracted from clinical isolates and transformants by sonication and were characterized by IEF (Flat Bed Apparatus, FBE 3000, Pharmacia Fine Chemicals, Uppsala, Sweden) according to the manufacturer’s procedure. Gels were developed using 0.5 mM nitrocefin (Becton-Dickinson, Oxford, UK). TEM-1 (pI = 5.4), SHV-5 (pI = 8.2) and OXA-4 (pI = 7.5) enzymes were used as controls.

Molecular detection of antibiotic resistance genes and integrons

The β-lactamase-encoding genes blaTEM and blaCTX-M (universal and group 1-specific), the aminoglycoside resistance genes aac3-IlA and aac6’-Ib, and the plasmid-mediated quinolone resistance gene qnr were sought by published PCR methods.4,10–12 blaOXA-1-like was sought with the primer pair 5’-GGA TAA AAC CCC CAA AGG AA-3’ and 5’-TGC ACC AGT TTT CCC ATA CA-3’ (PCR conditions: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 25 s, 52°C for 40 s and 72°C for 50 s; final elongation at 72°C for 6 min). Representative isolates and transformants were also screened by PCR for class 1–3 integrons13 and tetracycline resistance genes, including tet(A) and tet(B).14

Sequencing

Both strands of the bla and aac6’-Ib genes, repF replicons and the integron were sequenced with a Beckman CEQ 8000 Genetic Analysis System (Beckman Coulter, High Wycombe, UK). Sequences were edited with Bionumerics software (Applied Maths, Kortrijk, Belgium).

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RNA extraction and reverse transcriptase PCR (RT–PCR)

Total RNA from three representatives of each strain (A and D) was extracted with the RNeasy® Mini Kit (Qiagen, Crawley, UK) and was subjected to reverse-transcription using a Moloney-Murine Leukaemia Virus Reverse Transcriptase, according to the manufacturer’s procedure (Invitrogen, Paisley, UK). The method was used to assess expression of blaCTX-M-15 and blaOXA-1.

Southern blotting and hybridization

A digoxigenin-labelled probe derived from the blaCTX-M-15 gene sequence was produced using the primer pair MA1/MA2 according to the manufacturer’s kit procedure (Roche Diagnostics, Lewes, UK). Plasmid extracts were transferred from agarose gels to nylon membranes using a vacuum blotter (Amersham Biosciences, Buckinghamshire, UK). Hybridization of the membrane with the digoxigenin-labelled probe was performed overnight at 70°C and hybrids were detected with alkaline phosphatase linked to anti-digoxigenin Fab fragments, following addition of the colorimetric substrate NBT/BCIP [nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (Roche Diagnostics)].

Results and discussion

All clinical isolates of strains A and D were resistant to third-generation cephalosporins, with MICs of these agents reduced by clavulanic acid (Table 1). These isolates were also resistant to co-amoxiclav, to most aminoglycosides (except gentamicin in the case of strain A isolates), and to ciprofloxacin, tetracycline, trimethoprim and sulfamethoxazole. They remained susceptible to cefoxitin, nitrofurantoin, fosfomycin and carbapenems. IEF showed three β-lactamases, with pI values of 5.4, 7.4 and 8.6, to be expressed in different combinations; PCR and DNA sequencing showed that blaTEM-1, blaOXA-1 and blaCTX-M-15 genes, respectively, encoded these enzymes. All three enzymes were consistently expressed by the three strain D isolates examined. All 11 strain A isolates expressed CTX-M-15 and OXA-1, although CTX-M-15 was difficult to detect by IEF due to low expression, presumed to be contingent upon an IS26 insertion element that lies between blaCTX-M-15 and its normal promoter. Of the 11 strain A isolates, 6 produced TEM-1 enzyme; 5 did not, and lacked blaTEM. Lower CTX-M-15 expression by strain A than strain D was supported by RT–PCR data and by the lower cephalosporin MICs for strain A representatives and their transformants. Genetic linkage of blaCTX-M-15 with blaOXA-1 and, where present, blaTEM-1 was demonstrated by the co-transformation of these genes on single plasmids into E. coli DH5α from both strain A and strain D donors. Plasmid extraction and hybridization studies using the transformants showed that blaCTX-M-15 was located on ~150 and 70 kb plasmids in strains A and D, respectively; the absence of TEM-1 from 5/11 strain A isolates was not associated with a visible decrease in size of the 150 kb plasmids transferred. The 70 kb plasmid from strain D was conjugal, with a transfer frequency of 7.6×10^-6 per donor cell; conjugal transfer of cefotaxime resistance from strain A into E. coli J53-2 was not achieved in three separate attempts.

In addition to the bla genes, the plasmid from strain D encoded resistance to gentamicin, tobramycin and tetracycline, and reduced susceptibility to amikacin; the plasmid from strain A behaved similarly except that it also encoded resistance to...
Characterization of plasmids from multiresistant *E. coli*

Table 1. MICs for representative strain A and strain D isolates and their transformants (mg/L)

<table>
<thead>
<tr>
<th>Plasmid donors</th>
<th>Recipient</th>
<th>Transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Co-amoxiclav</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Cefotaxime/clavulanate</td>
<td>≤0.06</td>
<td>0.25</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Ceftazidime/clavulanate</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Cefepime</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>Meropenem</td>
<td>≤0.06</td>
<td>≤0.06</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>≤0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&gt;32</td>
<td>&gt;32</td>
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<tr>
<td>Amikacin</td>
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<td>8</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.5</td>
<td>&gt;32</td>
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<tr>
<td>Tobramycin</td>
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<td>&gt;32</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

trimethoprim and reduced susceptibility to sulfamethoxazole and did not confer gentamicin resistance. Although frank resistance to ciprofloxacin was not transferred, both plasmids conferred at least a 4-fold increase in the MIC, as shown by both Etest and agar dilution methods.

Genetic analyses revealed the aminoglycoside resistance genes $aac(6\text{-}Ib)-cr$ of strain A, correlating with their retained susceptibility to gentamicin, which is a substrate for AAC3 of strain D, whereas only the latter gene was detected in the transformants of strain D. In contrast, both strains had other non-plasmid-mediated quinolone resistance mechanisms.

The strain A plasmid, but not that from strain D, also carried a class 1 integron containing the streptomycin and trimethoprim resistance gene cassettes *addA5* and *dfr17*, correlating with trimethoprim resistance among its transformants; both the resistance and the *dfr17* gene were absent from transformants of strain D, although isolates of the strain itself were resistant.

The presence of *sul1* at the 3′ conserved sequence of the integron explains the higher sulfamethoxazole MICs among transformants of strain A than those of strain D. Tetracycline resistance in both strains was explicable by the presence of *tet(A)*.

Both strain A and D plasmids belonged to the FII incompatibility group, suggesting that they are related. In addition, the strain A plasmid harboured a second replicon (FIA), which was absent from the smaller strain D plasmid. This was tentatively proposed that the strain A plasmid may have arisen from the fusion of an FIA plasmid with a more ancestral FII plasmid, which may be partly identical to that of strain D. The class 1 integron, only detected in the plasmid from strain A, may be part of the FIA fragment, whereas the other resistances, including the *bla* genes, *tet(A)* and *aac(6\text{-}Ib)-cr*, may lie in the FII fragment. If so, the fusion event must post-date migration of the *bla* gene to mobile DNA and the evolution of a quinolone-recognizing AAC(6\text{-}Ib)-cr, which was not transferred, both plasmids conferred at least 4-fold increase in the MIC, as shown by both Etest and agar dilution methods.

Genetic analyses revealed the aminoglycoside resistance genes *aac(6\text{-}Ib)-cr* to be carried by the plasmid from strain D, whereas only the latter gene was detected in the transformants of strain A, correlating with their retained susceptibility to gentamicin, which is a substrate for AAC3. This was probably due to the *aac(6\text{-}Ib)-cr* fragment, whereas the other resistances, including the *bla* genes, *tet(A)* and *aac(6\text{-}Ib)-cr*, may lie in the FII fragment. If so, the fusion event must post-date migration of the *bla* gene to mobile DNA and the evolution of a quinolone-recognizing AAC(6\text{-}Ib)-cr, which was absent from the smaller strain D plasmid. It is tentatively proposed that the strain A plasmid may have arisen from the fusion of an FIA plasmid with a more ancestral FII plasmid, which may be partly identical to that of strain D. The class 1 integron, only detected in the plasmid from strain A, may be part of the FIA fragment, whereas the other resistances, including the *bla* genes, *tet(A)* and *aac(6\text{-}Ib)-cr*, may lie in the FII fragment. If so, the fusion event must post-date migration of the *bla* gene to mobile DNA and the evolution of a quinolone-recognizing AAC(6\text{-}Ib)-cr, which was absent from the smaller strain D plasmid. It is tentatively proposed that the strain A plasmid may have arisen from the fusion of an FIA plasmid with a more ancestral FII plasmid, which may be partly identical to that of strain D. The class 1 integron, only detected in the plasmid from strain A, may be part of the FIA fragment, whereas the other resistances, including the *bla* genes, *tet(A)* and *aac(6\text{-}Ib)-cr*, may lie in the FII fragment. If so, the fusion event must post-date migration of the *bla* gene to mobile DNA and the evolution of a quinolone-recognizing AAC(6\text{-}Ib)-cr, which was absent from the smaller strain D plasmid. It is tentatively proposed that the strain A plasmid may have arisen from the fusion of an FIA plasmid with a more ancestral FII plasmid, which may be partly identical to that of strain D. The class 1 integron, only detected in the plasmid from strain A, may be part of the FIA fragment, whereas the other resistances, including the *bla* genes, *tet(A)* and *aac(6\text{-}Ib)-cr*, may lie in the FII fragment. If so, the fusion event must post-date migration of the *bla* gene to mobile DNA and the evolution of a quinolone-recognizing AAC(6\text{-}Ib)-cr, which was absent from the smaller strain D plasmid. It is tentatively proposed that the strain A plasmid may have arisen from the fusion of an FIA plasmid with a more ancestral FII plasmid, which may be partly identical to that of strain D. The class 1 integron, only detected in the plasmid from strain A, may be part of the FIA fragment, whereas the other resistances, including the *bla* genes, *tet(A)* and *aac(6\text{-}Ib)-cr*, may lie in the FII fragment. If so, the fusion event must post-date migration of the *bla* gene to mobile DNA and the evolution of a quinolone-recognizing AAC(6\text{-}Ib)-cr, which was absent from the smaller strain D plasmid. It is tentatively proposed that the strain A plasmid may have arisen from the fusion of an FIA plasmid with a more ancestral FII plasmid, which may be partly identical to that of strain D. The class 1 integron, only detected in the plasmid from strain A, may be part of the FIA fragment, whereas the other resistances, including the *bla* genes, *tet(A)* and *aac(6\text{-}Ib)-cr*, may lie in the FII fragment.
including genetic linkage of the three β-lactamase genes, blaCTX-M-15, blaOXA-1 and blaTEM-1, as previously published for CTX-M-15-harbouring plasmids such as pC15-1a (GenBank AY458016) and pCTX15, found in E. coli isolates from Canada and India, respectively.16

Acknowledgements

We wish to thank AstraZeneca for their financial support and Dr Katie Hopkins, Laboratory of Enteric Pathogens, Health Protection Agency, Centre for Infections, for her help in plasmid incompatibility grouping. Part of this work was presented at the 15th ECCMID (P471), Copenhagen, Denmark (April 2005).

Transparency declarations

None to declare.

References