Plasmid typing and genetic context of AmpC β-lactamases in Enterobacteriaceae lacking inducible chromosomal ampC genes: findings from a Spanish hospital 1999–2007

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Objectives: To gain insights into ampC transmission between bacterial strains.

Methods: We examined the genetic context of 117 acquired ampC genes from 27119 Enterobacteriaceae collected between 1999 and 2007. Plasmid analysis was carried out by PCR-based replicon or relaxase typing, S1-PFGE and Southern hybridization. I-CeuI/PFGE was used for isolates not characterized by plasmid analysis. PCR reactions were used to map the genetic organization of the ampC genes.

Results: Among the isolates studied, 81.2% of ampC genes were located on plasmids of known Inc/MOB groups, 7.7% were chromosomally located and 11.1% were not determined. A/C, I1 and K were the most commonly found replicons in plasmids carrying blaCMY-2, while L/M replicons were associated with blaDHA-1. blaACC-1 was linked to I1 and MOBF11 plasmids; blaCMY-27 was associated with IncF and MOBF12 plasmids; the plasmid carrying blaCMY-25 could not be typed, and blaCMY-40 was chromosomally located. All 87 isolates carrying blaCMY-2, blaCMY-4, blaCMY-25, blaCMY-27, blaCMY-40 or blaACC-1 displayed the transposon-like structures IS6301/ΔIS6301-blaCMY-blac-sugE or ΔIS6301-blaACC-1-gdha. The most prevalent structure in blaDHA-1 (93.3% of cases) was identical to that described in the Klebsiella pneumoniae pTN60013 plasmid. Remarkably, in three isolates containing chromosomal blaCMY-2, this gene was mobilized by conjugation.

Conclusions: Although plasmids are the main cause of the rapid dissemination of ampC genes among bacteria, we need to be aware that other mobile genetic elements such as integrative and conjugative elements (ICEs) can be involved in the mobilization of these genes.

Keywords: incompatibility groups, relaxases, mobile genetic elements, resistance gene dissemination

Introduction

AmpC β-lactamases confer resistance to most β-lactams except cefepime and carbapenems. Although most of the AmpC enzymes are intrinsic and chromosomally encoded, some are also found on plasmids. This is due to the action of mobile genetic elements (MGEs) in several species that can capture these chromosomal genes and transfer them into mobile and/or conjugative plasmids, and subsequently disseminate them into many bacterial species that naturally lack these genes. As plasmids are the principal vehicles for the dissemination of a great variety of resistance genes, their study and understanding is critical for reversing the increasing trend in antibiotic resistance rates worldwide. PCR-based replicon typing (PBRT) is currently the method of choice for plasmid characterization in clinically relevant bacteria. However, this method has several limitations in identifying divergent or novel replicons. A new method based on the characterization of plasmid-encoded relaxases and covering a wider diversity of transmissible plasmids from γ-proteobacteria has recently been proposed.
There is little information regarding the association of plasmid-mediated ampC genes with specific plasmid families. The few reports to date are mainly based on biased collections (e.g. only one ampC gene reported, or a single host studied) and mainly focused on blaCMY-2, while reports on other ampC genes such as blaDHA-1 and blaACC-1 are either scarce or non-existent. The aim of this study was to determine the plasmid families and the genetic loci involved in the dissemination of acquired ampC genes into 117 Enterobacteriaceae lacking chromosomal AmpC enzymes. These isolates were recovered at Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) over a 9-year period (1999–2007). The predominantly acquired AmpC β-lactamase was found to be CMY-2 (n=78), followed by DHA-1 (n=30). Less commonly found enzymes were ACC-1 (n=3) and CMY-2 variants [CMY-4 (n=2), CMY-27 (n=2), CMY-25 (n=1) and CMY-40 (n=1)].

Methods

Bacterial isolates

A total of 27119 isolates of Enterobacteriaceae lacking inducible chromosomal AmpC enzymes were investigated between 1999 and 2007. The criteria for selecting putative acquired AmpC producers have been previously described. Among these isolates, 117 carried acquired ampC genes, including 75 Escherichia coli, 20 Proteus mirabilis, 16 Klebsiella pneumoniae, 4 Klebsiella oxytoca and 2 Salmonella enterica. Clonal relationships were established for one cluster of five P. mirabilis isolates, three clusters of two identical E. coli isolates and one cluster of two identical K. pneumoniae isolates.

Conjugation experiments

Conjugation assays were performed using a broth mating method at 37°C. A modified E. coli HB101 strain, UA6190 (aminoglycosides and rifampicin resistant), was used as the recipient strain. This recipient also expresses a green fluorescent protein (GFP) marker, integrated in the chromosome by the suicide mini-transposon delivery plasmid pAG408 together with two aminoglycosides resistance genes as described previously. Transconjugants were selected on Luria–Bertani (LB) agar supplemented with ceftazidime (10 mg/L) and rifampicin (100 mg/L), and were exposed to UV illumination to check for GFP fluorescence. The frequency of transfer was expressed as the ratio of transconjugants to total recipient cells.

Susceptibility testing of transconjugants

Disc diffusion susceptibility tests were performed on transconjugant strains, according to CLSI guidelines, using commercially available Neo-Sensitabs discs (Rosco Diagnostica S/A).

PBRT

PBRT was used to identify the major incompatibility (Inc) groups of plasmids present in transconjugants, or in parental strains when conjugation did not occur. When ampC genes were not associated with any of the Inc groups obtained by PBRT, CoE, IncU and IncP plasmids were tested. Template DNA was prepared by extraction of total DNA using the GenElute Bacterial Genomic DNA kit (Sigma).

Characterization of the relaxase MOB family

A PCR-based MOB amplification method was performed to identify the relaxase MOB family of plasmids carrying the ampC genes that could not be characterized by PBRT. Briefly, this new plasmid characterization methodology classifies plasmids according to their mob region; this encodes the plasmid relaxase, which is found in all conjugative and mobilizable plasmids. Conjugative plasmids can be classified into six MOB families (MOBα, MOBβ, MOBγ, MOBδ, MOBε and MOBζ) based on the amino acid sequence of their relaxases. Subgroups of five relaxase MOB families prevalent in the γ-proteobacteria (MOBα, MOBβ, MOBγ, MOBδ and MOBζ) were studied. When the relaxase was the only determinant obtained from plasmids mobilizing ampC genes, amplicons obtained from the MOB PCR were purified and sequenced. Primers and PCR conditions used to amplify these relaxases are listed in Table S1 (available as Supplementary data at JAC Online).

Plasmid profiles and Southern blot analysis

When possible, plasmid analysis was carried out in the transconjugant or donor strains by DNA linearization with S1 nuclease followed by PFGE as previously described. Plasmid sizes were estimated using Fingerprinting II Informatix software (Bio-Rad). DNA was transferred from S1-PFGE gels onto nylon membranes by Southern blotting. Purified DNA products obtained from the PCR of ampC genes, blaESBL genes, Inc group or MOB relaxase amplicons were used as probes for hybridization of the S1-PFGE blots. These probes were labelled with the Amersham ECL Direct Nucleic Acid Labelling and Detection Systems, according to the manufacturer’s instructions (GE Healthcare).

I-CeuI analysis

The chromosomal locations of ampC and blaESBL genes were investigated by digestion of the entire DNA with I-CeuI, followed by PFGE. This technique was applied to isolates that could not be characterized by the methods mentioned above.

Detection of the flanking regions of acquired ampC genes

An overlapping PCR strategy was used to map the regions surrounding these genes. The regions surrounding ampC genes most frequently described in the literature were explored. In the case of blaCMY-2, blaCMY-4, blaCMY-25, blaCMY-27, blaCMY-40, the presence of IScp1, blc and sugE genes was studied. As truncated versions of IScp1 (at either the 3’ or 5’ end) have been described, several primers were used to explore this region. For blaCMY-2, searched genes were IScp1 (orf513), ISc26, orf2 (conserved region of unknown function in Morganella species), ampr, qacEΔ1 and sul1; for blbAcc-1, searched genes were ISc26, IScp1 and gdh. Primers and PCR conditions are listed in Table S1.

Results

Conjugation experiments

All 117 isolates from the collection were studied. Among these, conjugative transfer of the ampC gene was detected for 97 isolates (82.9%). Conjugation frequencies ranged from 10⁻² to 10⁻⁸. Conjugation experiments involving CMY-4, CMY-25 and CMY-40 donors did not produce any transconjugants, despite testing under several different conjugation conditions (data not shown).

Susceptibility testing

Most donor strains showed high levels of resistance to all non-β-lactam antimicrobial agents tested (Table 1). However, the
and chloramphenicol were mainly present in isolates carrying most antibiotics tested. Resistance to sulphonamides, tetracycline isolates carrying ACC-1, CMY-25 and CMY-27 were susceptible to played higher rates of resistance to non-

The first approaches to characterize plasmids carrying

Plasmid characterization

The first approaches to characterize plasmids carrying *ampC* genes were PBRT and Southern hybridization using the amplicons obtained by PBRT as probes. Eleven of the 18 replicons tested, including I1, L/M, N, FIA, FIB, FIC, A/C, K, B/O and F, were involved in the dissemination of *ampC* genes. The most representative, alone or with other replicons, were A/C (n = 30), L/M (n = 25), I1 (n = 25), K (n = 10) and the F group (n = 10) (Table 2). The *ampC* genes were also found in plasmids with two or more replicons in 15 isolates (13%). The multi-replicon combinations identified were I1+F (n = 3), I1+K (n = 2), A/C+FIB+F (n = 2), L/M+FIA (n = 2), L/M+FIC (n = 2), L/M+N (n = 1), A/C+I1 (n = 1) and CoE+MOBP11 (n = 1) (Table 2). Characterization of plasmids carrying *ampC* gene was possible in 91 isolates in this first step (78%).

As a second approach, the remaining 26 non-typeable isolates were tested using PCR for CoIE, IncU and IncR replicons and for the relaxase MOB family. None of the isolates was positive for IncU or IncR plasmids. Hybridization with the CoIE replicons and the MOB relaxase amplicons found in these isolates allowed us to characterize another four plasmids carrying *ampC* genes: two MOBP11 plasmids, one MOBP12 plasmid and one co-integrate of a CoIE-like plasmid and a MOBP11 plasmid (Table 2). MOBP11 sequencing revealed that one case was compatible with the relaxase of the R46 (IncN-like) plasmid, while a novel MOBP11 relaxase was found for the other case (submitted to GenBank under accession number F421285). The translated sequence showed 79% amino acid identity (44/56) to TraI of IncN plasmid R46, and 51% (31/61) to TraI of pCT14. Sequencing of the MOBP11 and MOBP12 amplicons revealed relaxases identical to TraI of the IncP-1α plasmid RP4, and to NiK of the IncIα plasmid R64, respectively.

The 95 characterized strains showed that *ampC* genes were generally located on large plasmids of various sizes. The predominant plasmids present in the 78 strains carrying CMY-2, or CMY-2 plus an extended-spectrum *β*-lactamase (ESBL), belonged to Inc groups A/C (33%), I1 (23%) and K (10%); *blaCMY-2* was also found in multi-replicon plasmids (14%) (Table 2). On the other hand, all but one of the plasmids associated with DHA-1 (or DHA-1 plus ESBL) belonged to the L/M group. In most cases, L/M was the only replicon present (67%), or it was present in multi-replicon plasmids (17%). Genes encoding ACC-1, CMY-4 and CMY-27 were carried by IncI1, IncF, MOBP11 (an IncN-like and a novel relaxase) or MOBP12 (IncI1-like relaxase) plasmids.

No differences were observed in plasmid content among clonally related isolates, except for the cluster of two *E. coli* containing *blaCMY-27*. The *ampC* gene was located on an IncF plasmid in one case, while in the other case it was located on a MOBP12 plasmid.

The remaining 22 isolates were non-typeable by the methods used: (i) in nine isolates, no plasmids were observed in the S1-PFGE gel, or the plasmids present did not hybridize with the *ampC* gene; (ii) in three isolates, plasmids carrying *ampC* genes were not solved by PBRT or by relaxase-typing methods; (iii) in six isolates, multiple hybridization bands with *ampC* and other replicon probes (four *blaCMY-2* and two *blaDHA-1*) were found (when total DNA from several independent transconjugant colonies was analysed by S1-PFGE and hybridization with *ampC*

<table>
<thead>
<tr>
<th>Non-β-lactam antibiotics (% of resistant isolates)</th>
<th>Non-β-lactam antibiotics (% of resistant isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmpC enzymes</td>
<td>D or Tc (n)</td>
</tr>
<tr>
<td>CMY-2b</td>
<td>D (78)</td>
</tr>
<tr>
<td></td>
<td>Tc (66)</td>
</tr>
<tr>
<td>DHA-1c</td>
<td>D (30)</td>
</tr>
<tr>
<td></td>
<td>Tc (26)</td>
</tr>
<tr>
<td>ACC-1</td>
<td>D (3)</td>
</tr>
<tr>
<td></td>
<td>Tc (3)</td>
</tr>
<tr>
<td>CMY-27</td>
<td>D (2)</td>
</tr>
<tr>
<td></td>
<td>Tc (2)</td>
</tr>
<tr>
<td>CMY-4d</td>
<td>D (2)</td>
</tr>
<tr>
<td></td>
<td>D (1)</td>
</tr>
<tr>
<td>CMY-40d</td>
<td>D (1)</td>
</tr>
</tbody>
</table>

NAL, nalidixic acid; CIP, ciprofloxacin; SSS, sulphonamides; TMP, trimethoprim; TET, tetracycline; CHL, chloramphenicol; D, clinical strain; Tc, transconjugant.

aWhen the number of strains is less than 10, the resistant isolates are expressed as a rather than %.

bTwo of these isolates also harboured an ESBL; the plasmid mobilizing *ampC* was conjugative in one case.

cFive of these isolates also harboured an ESBL; plasmids mobilizing these genes were conjugative in all cases.

dTransconjugants were not obtained in isolates carrying CMY-4, CMY-25 or CMY-40.
probes, we observed several patterns: one or two hybridization bands of different sizes depending on the specific transconjugant (data not shown); and (iv) four isolates were degraded during S1-PFGE and therefore could not be characterized (Table 2).

I-CeuI/PFGE analysis was applied to all the unsolved isolates except those that were degraded during S1-PFGE. A chromosomal location was confirmed in the nine isolates (eight P. mirabilis with CMY-2 and one E. coli with CMY-40) without ampC plasmid hybridization signals in the S1-PFGE gel. The eight P. mirabilis strains were not clonally related. Four of these isolates were positive for MOBH12 amplicons, compatible with the presence of an SXT/R391-like element; for three of these P. mirabilis isolates containing a chromosomal copy of blaCMY-2, the gene had been mobilized by conjugation.

Two of the three non-characterized plasmids carrying ampC genes, and all isolates showing multiple hybridization bands

\[ \text{Table 2. Plasmid families associated with acquired AmpC } \beta \text{-lactamases in Enterobacteriaceae} \]

<table>
<thead>
<tr>
<th>Enzyme (n)</th>
<th>Replicons/relaxases</th>
<th>Isolates (n)</th>
<th>Strains (n)</th>
<th>Sizes (kb)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMY-2/CMY-2+ESBL (78)</td>
<td>A/C</td>
<td>E. coli (15), K. pneumoniae (2), P. mirabilis (8), S. enterica (1)</td>
<td>26</td>
<td>~95 to 365</td>
<td>33</td>
</tr>
<tr>
<td>I1</td>
<td>E. coli (16), P. mirabilis (1), S. enterica (1)</td>
<td>18</td>
<td>~80 to 350</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>E. coli (7), P. mirabilis (1)</td>
<td>8</td>
<td>~80 to 115</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>I1+K</td>
<td>E. coli (2)</td>
<td>2</td>
<td>~90 and 95</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>I1+F</td>
<td>E. coli (3)</td>
<td>3</td>
<td>~85 to 135</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>AC+FIB+F</td>
<td>E. coli (2)</td>
<td>2</td>
<td>~160 and 340</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>AC+FIA+FIB</td>
<td>E. coli (1)</td>
<td>1</td>
<td>~300</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AC+I1</td>
<td>E. coli (1)</td>
<td>1</td>
<td>~415</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>E. coli (1)</td>
<td>1</td>
<td>~190</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>ColE+MOBP12</td>
<td>P. mirabilis</td>
<td>1</td>
<td>~100</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>chromosomal location</td>
<td>P. mirabilis</td>
<td>8</td>
<td>—</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

| DHA-1/DHA-1+ESBL (30) | L/M | E. coli (8), K. pneumoniae (9), K. oxytoca (3) | 20 | ~70 to 295 | 67 |
| L/M+FIA | E. coli (2) | 2 | ~155 and 170 | 7 |
| L/M+FIC | K. pneumoniae (1), K. oxytoca (1) | 2 | ~110 and 150 | 7 |
| N | E. coli (1) | 1 | ~50 | 3 |
| L/M+N | P. mirabilis (1) | 1 | ~105 | 3 |
| non-typeable | E. coli (2) | 2 | ~75 to 310 | 7 |
| degraded | E. coli (2) | 2 | — | 7 |
| ACC-1 (3) | I1 | E. coli (1) | 1 | ~95 | 33 |
| MOB11 | E. coli (1), K. pneumoniae (1) | 2 | ~65 and 75 | 67 |
| CMY-4 (2) | F | E. coli (1) | 1 | ~120 | 50 |
| non-typeable | E. coli (1) | 1 | ~40 | 50 |
| CMY-27 (2) | F | E. coli (1) | 1 | ~75 | 50 |
| MOB12 | E. coli (1) | 1 | ~250 | 50 |
| CMY-25 (1) | non-typeable | K. pneumoniae (1) | 1 | ~380 | 100 |
| CMY-40 (1) | chromosomal location | E. coli (1) | 1 | — | 100 |

*F* includes all the IncF plasmids amplified by F-simplex PCR using the PBRT method; MOB11 belongs to the MOBrelaxase family (includes IncP-1 plasmids); MOB12 belongs to the MOBrelaxase family (includes IncI, K and B/O); MOB11 belongs to the MOBrelaxase family (includes IncN and IncW).

*One* P. mirabilis also harboured blaCTX-M-2 (also located on the chromosome). These isolates are not clonally related. An SXT/R391-like element was involved in the mobilization of blaCMY-2 genes in seven out of the eight P. mirabilis.

*The plasmid carrying blaCMY-2 in one E. coli could not be typed either by PBRT or by relaxase typing. This isolate also carried copies of blaCMY-2 on the chromosome. The remaining two E. coli showed multiple hybridization bands with the ampC gene and the replicon probes involved, and copies on the chromosome.

*One* K. pneumoniae also harboured blaCTX-M-1 on the plasmid containing blaCMY-2. Both showed multiple hybridization bands with the ampC gene and the replicon probes involved, and copies on the chromosome.

*Two* E. coli also harboured blaCTX-M-14 genes on different plasmids belonging to the IncI group (~90 kb).

*One* K. pneumoniae also harboured blaCTX-M-15 genes on the same IncI/M plasmid (~170 kb).

*One* K. pneumoniae also harboured blaCTX-M-15 genes on the same cointegrate IncI/M+FIC (~155 kb).

*Both* isolates showed multiple hybridization bands with blaCTX-M-2 and L/M probes. They also had copies on the chromosome.

*The plasmid carrying blaCMY-25 could not be typed either by PBRT or by relaxase typing. This isolate also carried copies of blaCMY-25 on the chromosome.
with the replicon and the ampC probes, also had copies of the ampC gene located on the chromosome.

In three of the seven isolates with ampC and bla\textsubscript{ESBL} genes, both genes were located on the same plasmid; in two isolates, bla\textsubscript{ESBL} genes (one bla\textsubscript{CTX-M-2} and one bla\textsubscript{CTX-M-15}) were located on the chromosome; in the remaining two, ampC and bla\textsubscript{ESBL} genes were located on different plasmids.

**Detection of the flanking regions of acquired ampC genes**

**Genetic organization of bla\textsubscript{CMY-2} and its derivatives**

ISEcp1 and the genes blc (outer membrane lipoprotein) and sugE (drug efflux channel) were found in all the screened isolates (Figure 1). ISEcp1 is responsible for the transfer of the bla\textsubscript{CMY-2}\textsubscript{-like}–blc-sug\textsubscript{E} region from the chromosome of Citrobacter freundii to a plasmid. However, truncation of ISEcp1 was observed in 54.8% (46/84) of the isolates. ISEcp1 was truncated at the 5′ end in 28.6% (24/84) of these, as amplicons were not obtained when using the a–b or a–d primer combinations, whilst c–d primers produced amplification products (Table S1 and Figure 1). In the remaining 26.2% (22/84) of the isolates, ISEcp1 was truncated at the 3′ end; in these cases, a–d primers successfully amplified a product of 1584 bp instead of the 2164 bp expected.

**Genetic context of AmpC β-lactamases in Enterobacteriaceae**

In this work we characterized the genetic context of the largest collection to date of plasmid-mediated AmpC β-lactamases in Enterobacteriaceae lacking inducible chromosomal AmpC enzymes, describing the widest variety of ampC genes in a single study. The prevalence of AmpC enzymes is increasing, mainly due to the transfer of these genes into mobilizable and conjugative plasmids.

Our results showed a close relationship between each ampC gene and the plasmid involved. Plasmids belonging to the A/C and 11 Inc groups are the most frequently reported bla\textsubscript{CMY-2} Carriers. In the present study, A/C and 11 Inc groups were also the most prevalent plasmids carrying bla\textsubscript{CMY-2}, followed by IncK plasmids. Although the bla\textsubscript{OXA-1} gene has mainly been associated with IncFII plasmids in previous reports, our collection exhibited a clear association with IncI/M replicons. Several recent studies have also described the association of bla\textsubscript{OXA-1} with IncI/M plasmids. This new trend could be explained by a direct link between bla\textsubscript{OXA-1} and qnr\textsubscript{B} to the IncI/M plasmids identified in the present collection. This association could explain why the 61.5% of transconjugants carrying bla\textsubscript{OXA-1} displayed reduced susceptibility to nalidixic acid.

The fact that PBRT failed to detect two out of three bla\textsubscript{ACC-1}-carrying plasmids of our collection and that one of the MOB\textsubscript{F11} relaxases found has a novel sequence suggests that the bla\textsubscript{ACC-1} gene could be carried by new backbone undetected by PBRT.

Plasmid associations of the recently-described bla\textsubscript{CMY-25}, bla\textsubscript{CMY-27} were expected to be similar to those obtained for bla\textsubscript{CMY-2}, as is the case for other bla\textsubscript{CMY-2} variants. However, only one bla\textsubscript{CMY-27} was carried by an IncI\textsubscript{a} plasmid, as identified by the relaxase-typing method. One bla\textsubscript{CMY-25} and one bla\textsubscript{CMY-27} were carried by IncF plasmids, which are less commonly found associated with bla\textsubscript{CMY-2} and usually appear in multi-replicon plasmids; the plasmid carrying bla\textsubscript{CMY-25} found in a K. pneumoniae isolate could not be solved either by PBRT or by relaxase typing.

Among the remaining 22 isolates that were not solved by plasmid analysis, nine had the ampC gene chromosomally located, and six showed multiple hybridization bands with ampC and replicon probes. It is also remarkable that these six isolates also had copies of ampC located on the chromosome. A picture of broad gene dissemination should be kept in mind, where different hybridization bands were obtained from different transconjugants arising from the same donor. This suggests either that different co-integrations from plasmid harbouring ampC genes are formed during conjugation, or that a highly active MGE is involved in the ampC genes – moving between different replicons in the donor strain. Further studies are needed to address this issue.

Moreover, in seven out of eight P. mirabilis where bla\textsubscript{CMY-2} was located on the chromosome, ampC genes were mobilized by SXT/R391-like integrative and conjugative elements (ICEs).

Furthermore, we analysed the regions surrounding the ampC genes. The genetic organization of bla\textsubscript{CMY-2} and its variants was highly conserved. All the isolates carried the transposon-like element ISEcp1 (ISEcp1\textsuperscript{Δ}ISEcp1-bla\textsubscript{CMY-2}-sug\textsubscript{E}). A well-conserved structure was also found in all isolates carrying bla\textsubscript{ACC-1} genes (ΔISEcp1-bla\textsubscript{ACC-1}-gdha). However, although IS26 is commonly related to the transmission of ACC-1 enzymes (and normally appears within ISEcp1), one of our isolates harboured this insertion sequence. The genetic organization of bla\textsubscript{OXA-1} was more variable. Mobilization of DHA-1 enzymes has been associated with

**Discussion**

In this work we characterized the genetic context of the largest collection to date of plasmid-mediated AmpC β-lactamases in Enterobacteriaceae lacking inducible chromosomal AmpC enzymes, describing the widest variety of ampC genes in a single study. The prevalence of AmpC enzymes is increasing, mainly due to the transfer of these genes into mobilizable and conjugative plasmids.

Our results showed a close relationship between each ampC gene and the plasmid involved. Plasmids belonging to the A/C and 11 Inc groups are the most frequently reported bla\textsubscript{CMY-2} Carriers. In the present study, A/C and 11 Inc groups were also the most prevalent plasmids carrying bla\textsubscript{CMY-2}, followed by IncK plasmids. Although the bla\textsubscript{OXA-1} gene has mainly been associated with IncFII plasmids in previous reports, our collection exhibited a clear association with IncI/M replicons. Several recent studies have also described the association of bla\textsubscript{OXA-1} with IncI/M plasmids. This new trend could be explained by a direct link between bla\textsubscript{OXA-1} and qnr\textsubscript{B} to the IncI/M plasmids identified in the present collection. This association could explain why the 61.5% of transconjugants carrying bla\textsubscript{OXA-1} displayed reduced susceptibility to nalidixic acid.

The fact that PBRT failed to detect two out of three bla\textsubscript{ACC-1}-carrying plasmids of our collection and that one of the MOB\textsubscript{F11} relaxases found has a novel sequence suggests that the bla\textsubscript{ACC-1} gene could be carried by new backbones undetected by PBRT.

Plasmid associations of the recently-described bla\textsubscript{CMY-25}, bla\textsubscript{CMY-27} were expected to be similar to those obtained for bla\textsubscript{CMY-2}, as is the case for other bla\textsubscript{CMY-2} variants. However, only one bla\textsubscript{CMY-27} was carried by an IncI\textsubscript{a} plasmid, as identified by the relaxase-typing method. One bla\textsubscript{CMY-25} and one bla\textsubscript{CMY-27} were carried by IncF plasmids, which are less commonly found associated with bla\textsubscript{CMY-2} and usually appear in multi-replicon plasmids; the plasmid carrying bla\textsubscript{CMY-25} found in a K. pneumoniae isolate could not be solved either by PBRT or by relaxase typing.

Among the remaining 22 isolates that were not solved by plasmid analysis, nine had the ampC gene chromosomally located, and six showed multiple hybridization bands with ampC and replicon probes. It is also remarkable that these six isolates also had copies of ampC located on the chromosome. A picture of broad gene dissemination should be kept in mind, where different hybridization bands were obtained from different transconjugants arising from the same donor. This suggests either that different co-integrations from plasmid harbouring ampC genes are formed during conjugation, or that a highly active MGE is involved in the ampC genes – moving between different replicons in the donor strain. Further studies are needed to address this issue.

Moreover, in seven out of eight P. mirabilis where bla\textsubscript{CMY-2} was located on the chromosome, ampC genes were mobilized by SXT/R391-like integrative and conjugative elements (ICEs).

Furthermore, we analysed the regions surrounding the ampC genes. The genetic organization of bla\textsubscript{CMY-2} and its variants was highly conserved. All the isolates carried the transposon-like element ISEcp1 (ISEcp1\textsuperscript{Δ}ISEcp1-bla\textsubscript{CMY-2}-sug\textsubscript{E}). A well-conserved structure was also found in all isolates carrying bla\textsubscript{ACC-1} genes (ΔISEcp1-bla\textsubscript{ACC-1}-gdha). However, although IS26 is commonly related to the transmission of ACC-1 enzymes (and normally appears within ISEcp1), one of our isolates harboured this insertion sequence. The genetic organization of bla\textsubscript{OXA-1} was more variable. Mobilization of DHA-1 enzymes has been associated with
Figure 1. Genetic organization of the \textit{ampC} genes. \textit{ampC} genes are represented by filled arrows, while the surrounding genes are represented by white arrows. Truncated genes are represented by disrupted arrows. (1.1) 45.2\% of the isolates showed an identical structure to pNF4656 (AY581207). (1.2) 28.6\% of the isolates showed ISEcp1 truncated at the 5’ end. (1.3) 26.2\% of the isolates showed ISEcp1 truncated at the 3’ end. (2.1) 93.3\% of the isolates showed an identical structure to pTN60013 (AJ971345). (2.2) One isolate showed an identical structure to pT498 (AY705809). (2.3) In one isolate, IS26 was not present either upstream or downstream of \textit{bla}_{\text{DHA-1}}. (3.1) All three isolates showed an identical structure to \textit{S. enterica} serovar Bareilly 60.50 (AY856832).
IS26 or class 1 integron-bearing ISCR1 elements. We found three similar structures (Figure 1), but ISCR1 was not involved in any of them. Structures I and II were identical to those described in the K. pneumoniae plasmids pTN60013 (AJ971345) and pT498 (AY705809), respectively. The most prevalent was structure I (93.3% of cases). IS26 and qacEΔ1 and sulI genes – the last two belong to the 3’ conserved sequence of class 1 integrons – were present in both structures. In the single isolate displaying structure III, only qacEΔ1 and sulI genes were found.

Although plasmids have been shown to be the main vehicles for the fast dissemination of ampC genes among clinically relevant bacteria, other MGEs may also play an important role in the marked increase in the prevalence of these enzymes. Further studies focusing not only on plasmids but also on other MGEs such as ICEs are needed to appreciate the complex processes involved in the dissemination of antibiotic resistance genes worldwide.

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Transparency declarations
None to declare.

Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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