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

Caterina Mata\(^1\), Elisenda Miró\(^1\), Andrés Alvarado\(^2\), M. Pilar Garcillán-Barcia\(^2\), Mark Toleman\(^3\), Timothy R. Walsh\(^3\), Fernando de la Cruz\(^2\) and Ferran Navarro\(^1\)*

\(^1\)Servei de Microbiologia, Hospital de la Santa Creu i Sant Pau, Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Institut d’Investigacions Biomèdiques Sant Pau, Barcelona, Spain; \(^2\)Departamento de Biología Molecular, Universidad de Cantabria e Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC), Cantabria, Spain; \(^3\)Section of Medical Microbiology, IIB, School of Medicine, Cardiff University, Heath Park, Cardiff, UK

*Corresponding author. Servei de Microbiologia, Hospital de La Santa Creu i Sant Pau, C/Sant Quinti 89, 08025 Barcelona, Spain. Tel: +34-935537297; Fax: +34-935537287; E-mail: fnavarro@santpau.cat

Received 22 March 2011; returned 20 June 2011; revised 15 August 2011; accepted 6 September 2011

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 \(bla\)CMY-2, while L/M replicons were associated with \(bla\)DHA-1. \(bla\)ACC-1 was linked to I1 and MOBF11 plasmids; \(bla\)CMY-27 was associated with IncF and MOBp12 plasmids; the plasmid carrying \(bla\)CMY-25 could not be typed, and \(bla\)CMY-40 was chromosomally located. All 87 isolates carrying \(bla\)CMY-2, \(bla\)CMY-4, \(bla\)CMY-25, \(bla\)CMY-27, \(bla\)CMY-40 or \(bla\)ACC-1 displayed the transposon-like structures IS\(Ecp1\)/\(IS\)Ecp1-blaCMY-blc-sugE or \(IS\)Ecp1-blaACC-1-gdha. The most prevalent structure in \(bla\)DHA-1 (93.3% of cases) was identical to that described in the \(Klebsiella pneumoniae\) pTN60013 plasmid. Remarkably, in three isolates containing chromosomal \(bla\)CMY-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 \(\beta\)-lactamases confer resistance to most \(\beta\)-lactams except cefepime and carbapenems.\(^3\) 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 mobilizable and/or conjugative plasmids, and subsequently disseminate them into many bacterial species that naturally lack these genes.\(^1\)–\(^5\)

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)\(^6\) 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.\(^1\)–\(^6\) A new method based on the characterization of plasmid-encoded relaxases and covering a wider diversity of transmissible plasmids from \(\gamma\)-proteobacteria has recently been proposed.\(^8\)
There is little information regarding the association of plasmid-mediated \( \text{ampC} \) genes with specific plasmid families. The few reports to date are mainly based on biased collections (e.g., only one \( \text{ampC} \) gene reported, or a single host studied)\(^7\) and mainly focused on \( \text{bla}_{\text{CMY-2}} \), while reports on other \( \text{ampC} \) genes such as \( \text{bla}_{\text{DHA-1}} \) and \( \text{bla}_{\text{ACC-1}} \) 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 \( \text{ampC} \) genes into 117 Enterobacteriaceae lacking chromosomal \( \text{ampC} \) 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 \( \beta \)-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)].\(^9\)

**Methods**

**Bacterial isolates**

A total of 27,119 isolates of Enterobacteriaceae lacking inducible chromosomal \( \text{ampC} \) enzymes were investigated between 1999 and 2007. The criteria for selecting putative acquired \( \text{ampC} \) producers have been previously described.\(^7\) Among these isolates, 117 carried acquired \( \text{ampC} \) genes, including 75 \( \text{Escherichia coli} \), 20 \( \text{Proteus mirabilis} \), 16 \( \text{Klebsiella pneumoniae} \), 4 \( \text{Klebsiella oxytoca} \) and 2 \( \text{Salmonella enterica} \). Clonal relationships were established for one cluster of five \( \text{P. mirabilis} \) isolates, three clusters of two identical \( \text{E. coli} \) isolates and one cluster of two identical \( \text{K. pneumoniae} \) isolates.\(^9\)

**Conjugation experiments**

Conjugation assays were performed using a broth mating method at 37°C. A modified \( \text{E. coli} \) 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 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,\(^14\) using commercially available Neo-Sensitabs discs (Rosco Diagnostica S/A).

**PBRT**

PBRT\(^6\) was used to identify the major incompatibility (Inc) groups of plasmids present in transconjugants, or in parental strains when conjugation did not occur. When \( \text{ampC} \) genes were not associated with any of the Inc groups obtained by PBRT, CoE, IncU and IncR plasmids were tested.\(^12\)

**Characterization of the relaxase MOB family**

A PCR-based MOB amplification method\(^8,13\) was performed to identify the relaxase MOB family of plasmids carrying the \( \text{ampC} \) genes that could not be characterized by PBRT. Briefly, this new plasmid characterization methodology classifies plasmids according to their \( \text{mob} \) region; this encodes the plasmid relaxase, which is found in all conjugative and mobilizable plasmids. Conjugative plasmids can be classified into six MOB families (\( \text{MOB} \text{R} \), \( \text{MOB} \text{P} \), \( \text{MOB} \text{B} \), \( \text{MOB} \text{C} \), \( \text{MOB} \text{D} \) and \( \text{MOB} \text{H} \)) based on the amino acid sequence of their relaxases. Subgroups of five relaxase MOB families prevalent in the \( \gamma \)-proteobacteria (\( \text{MOB} \text{R} \), \( \text{MOB} \text{P} \), \( \text{MOB} \text{C} \), \( \text{MOB} \text{D} \) and \( \text{MOB} \text{H} \)) were studied. When the relaxase was the only determinant obtained from plasmids mobilizing \( \text{ampC} \) 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.\(^14\) Plasmid sizes were estimated using Fingerprinting II InformatixTM software (Bio-Rad). DNA was transferred from S1-PFGE gels onto nylon membranes by Southern blotting. Purified DNA products obtained from the PCR of \( \text{ampC} \) genes, \( \text{bla}_{\text{EISL}} \) genes, \( \text{Inc} \) 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 \( \text{ampC} \) and \( \text{bla}_{\text{EISL}} \) genes were investigated by digestion of the entire DNA with I-CeuI, followed by PFGE.\(^15\) This technique was applied to isolates that could not be characterized by the methods mentioned above.

**Detection of the flanking regions of acquired \text{ampC} \text{ genes}**

An overapping PCR strategy was used to map the regions surrounding these genes. The regions surrounding \( \text{ampC} \) genes most frequently described in the literature were explored.\(^1,5,6-19\) In the case of \( \text{bla}_{\text{CMY-2}}, \text{bla}_{\text{CMY-4}}, \text{bla}_{\text{CMY-25}}, \text{bla}_{\text{CMY-27}}, \text{bla}_{\text{CMY-40}} \), the presence of \( \text{IscE} \text{p1} \), \( \text{blc} \) and \( \text{sugE} \) genes was studied. As truncated versions of \( \text{IscE} \text{p1} \) (at either the 3’ or 5’ end) have been described, several primers were used to explore this region. For \( \text{bla}_{\text{DHA-1}}, \text{bla}_{\text{CMY-40}}, \text{bla}_{\text{CMY-27}}, \text{bla}_{\text{CMY-25}} \) donors did not produce any transconjugants, despite testing under several different conjugation conditions (data not shown).

**Results**

**Conjugation experiments**

All 117 isolates from the collection were studied. Among these, conjugative transfer of the \( \text{ampC} \) 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-\( \beta \)-lactam antimicrobial agents tested (Table 1). However, the
Genetic context of AmpC β-lactamases in Enterobacteriaceae

Table 1. Non-β-lactam antibiotic susceptibility testing

<table>
<thead>
<tr>
<th>AmpC enzymes</th>
<th>D or Tc (n)</th>
<th>NAL</th>
<th>CIP</th>
<th>SSS</th>
<th>TMP</th>
<th>SXT</th>
<th>TET</th>
<th>CHL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMY-2</td>
<td>D (78)</td>
<td>76.9</td>
<td>47.4</td>
<td>73.1</td>
<td>43.6</td>
<td>39.7</td>
<td>80.7</td>
<td>59.0</td>
</tr>
<tr>
<td></td>
<td>Tc (66)</td>
<td>0.0</td>
<td>0.0</td>
<td>50.0</td>
<td>3.0</td>
<td>3.0</td>
<td>47.0</td>
<td>42.4</td>
</tr>
<tr>
<td>DHA-1</td>
<td>D (30)</td>
<td>76.7</td>
<td>60.0</td>
<td>46.6</td>
<td>46.7</td>
<td>36.7</td>
<td>43.3</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td>Tc (26)</td>
<td>61.5</td>
<td>0.0</td>
<td>15.4</td>
<td>15.4</td>
<td>7.7</td>
<td>11.5</td>
<td>0.0</td>
</tr>
<tr>
<td>ACC-1</td>
<td>D (3)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Tc (3)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CMY-27</td>
<td>D (2)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Tc (2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CMY-4</td>
<td>D (2)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>CMY-25</td>
<td>D (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CMY-40</td>
<td>D (1)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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

bWhen the number of strains is less than 10, the resistant isolates are expressed as n rather then %.

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

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

eTransconjugants were not obtained in isolates carrying CMY-4, CMY-25 or CMY-40.

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 CoE, IncU and IncR replicons and for the relaxase MOB family. None of the isolates was positive for IncU or IncR replicons. Hybridization with the CoE 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 CoE-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 FA421285). 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 NikB 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 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 blαCMY-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 blαDHA-1) were found (when total DNA from several independent transconjugant colonies was analysed by S1-PFGE and hybridization with ampC.
Table 2. Plasmid families associated with acquired AmpC β-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/CY-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></td>
<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>
</tr>
<tr>
<td></td>
<td>K</td>
<td>E. coli (7), P. mirabilis (1)</td>
<td>8</td>
<td>~80 to 115</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>I1 + K</td>
<td>E. coli (2)</td>
<td>2</td>
<td>~90 and 95</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>I1 + F</td>
<td>E. coli (3)</td>
<td>3</td>
<td>~85 to 135</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>A/C + FIB</td>
<td>E. coli (2)</td>
<td>2</td>
<td>~160 and 340</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>A/C + FIB + F</td>
<td>E. coli (1)</td>
<td>1</td>
<td>~300</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>A/C + I1</td>
<td>E. coli (1)</td>
<td>1</td>
<td>~415</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>ColE + MOBP11</td>
<td>P. mirabilis (1)</td>
<td>1</td>
<td>≈100</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>chromosomal</td>
<td>P. mirabilisb (8)</td>
<td>8</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>non-typeable</td>
<td>E. coli (3), K. pneumoniaiae (2)</td>
<td>5</td>
<td>~110 to 425</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>degraded</td>
<td>E. coli (2)</td>
<td>2</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>DHA-1/DHA-1 + ESBL (30)</td>
<td>L/M</td>
<td>E. coli (16), K. pneumoniae (9), K. oxytoca (3)</td>
<td>20</td>
<td>~70 to 295</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>L/M + FIA</td>
<td>E. coli (2)</td>
<td>2</td>
<td>~155 and 170</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>L/M + FIC</td>
<td>K. pneumoniaeb (1), K. oxytoca (1)</td>
<td>2</td>
<td>~110 and 150</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>E. coli (1)</td>
<td>1</td>
<td>~50</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>L/M + N</td>
<td>P. mirabilis (1)</td>
<td>1</td>
<td>~105</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>non-typeable</td>
<td>E. coli (2)</td>
<td>2</td>
<td>~75 to 310</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>degraded</td>
<td>E. coli (2)</td>
<td>2</td>
<td>—</td>
<td>7</td>
</tr>
<tr>
<td>ACC-1 (3)</td>
<td>I1</td>
<td>E. coli (1)</td>
<td>1</td>
<td>~95</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>MOBP11</td>
<td>E. coli (1), K. pneumoniae (1)</td>
<td>2</td>
<td>~65 and 75</td>
<td>67</td>
</tr>
<tr>
<td>CMY-4 (2)</td>
<td>F</td>
<td>E. coli (1)</td>
<td>1</td>
<td>~120</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>non-typeable</td>
<td>E. coli (1)</td>
<td>1</td>
<td>~40</td>
<td>50</td>
</tr>
<tr>
<td>CMY-27 (2)</td>
<td>F</td>
<td>E. coli (1)</td>
<td>1</td>
<td>~75</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>MOBP12</td>
<td>E. coli (1)</td>
<td>1</td>
<td>~250</td>
<td>50</td>
</tr>
<tr>
<td>CMY-25 (1)</td>
<td>non-typeable</td>
<td>K. pneumoniae (1)</td>
<td>1</td>
<td>~380</td>
<td>100</td>
</tr>
<tr>
<td>CMY-40 (1)</td>
<td>chromosomal</td>
<td>E. coli (1)</td>
<td>1</td>
<td>—</td>
<td>100</td>
</tr>
</tbody>
</table>

aF includes all the IncF plasmids amplified by F-simplex PCR using the PBRT method; MOBP11 belongs to the MOBP relaxase family (includes IncP-1 plasmids); MOBP12 belongs to the MOBP relaxase family (includes IncI, K and B/O); MOBF11 belongs to the MOBF relaxase family (includes IncN and IncW).
bOne 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.
cThe 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.
dOne K. pneumoniae also harboured blaCTX-M-15 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.
eTwo E. coli also harboured blaCTX-M-15 genes on different plasmids belonging to the IncI group (~90 kb).
fOne K. pneumoniae also harboured blaCTX-M-15 genes on the same IncI/M plasmid (~170 kb).
gOne K. pneumoniae also harboured blaCTX-M-15 genes on the same cointegrate IncI/M + FIC (~155 kb).
hBoth isolates showed multiple hybridization bands with blaCMY-2 and L/M probes. They also had copies on the chromosome.
iThe 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.

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 MOBP11 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...
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 blaESBL genes, 
both genes were located on the same plasmid; in two isolates, 
blaESBL genes (one blaCTX-M-2 and one blaCTX-M-15) were located 
on the chromosome; in the remaining two, ampC and blaESBL 
genes were located on different plasmids.

Detection of the flanking regions of acquired ampC genes

Genetic organization of blaCMY-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 
blaCMY-2-like-blc-sugE region from the chromosome of Citrobacter freundii to a plasmid.5 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 
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,2 describing the widest variety of ampC genes in a 
single study. The prevalence of AmpC enzymes is increasing,1,9,20 
mainly due to the transfer of these genes into mobilizable and 
conjugal 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 blaCMY-2 Car-
rriers.3,4,7,20–22 In the present study, A/C and 11 Inc groups were 
also the most prevalent plasmids carrying blaCMY-2, followed by 
IncK plasmids. Although the blaQH1 gene has mainly been 
associated with IncFII plasmids in previous reports,7,21,22 our col-
llection exhibited a clear association with IncI/M replicons. 
Several recent studies have also described the association of 
blaQH1 with IncL/M plasmids.24,25 This new trend could be 
explained by a direct link between blaQH1 and qnrB to the 
IncI/M plasmids identified in the present collection.26 This 
association could explain why the 61.5% of transconjugants 
carrying blaQH1 displayed reduced susceptibility to nalidixic acid.26 
The three blaACC-1 genes were carried by I1 (n = 1) and MOBf11 
(n = 2) plasmids. One of the MOBf11 relaxases had a sequence 
not previously reported. Data on plasmids carrying blaACC-1 are 
scarce. To our knowledge, the plasmid location of blaACC-1 has 
only been reported once, and the plasmid could not be typed 
by PBRT.23 Thus, this is the first time that plasmids carrying 
blaACC-1 have been typed.

The fact that PBRT also failed to detect two out of three 
blaACC-1-carrying plasmids of our collection and that one of the 
MOBf11 relaxases found has a novel sequence suggests that 
the blaACC-1 gene could be carried by new backbones undetected 
by PBRT.

Plasmid associations of the recently-described blaCMY-25, 
blaCMY-27 were expected to be similar to those obtained for 
blaCMY-2, as is the case for other blaCMY-2 variants.9 However, 
only one blaCMY-27 was carried by an IncKI plasmid, as identified 
by the relaxase-typing method. One blaCMY-27 and one blaCMY-25 
were carried by IncF plasmids, which are less commonly found 
associated with blaCMY-2 and usually appear in multi-replicon plas-
mids; the plasmid carrying blaCMY-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 
isolates also had copies of ampC located on the chromosome. 
A picture of broad gene dissemination should be kept in mind, 
since different hybridization bands were obtained from different 
transconjugants arising from the same donor. This suggests 
that either 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 blaCMY-2 was 
located on the chromosome, ampC genes were mobilized by SXT/ 
R391-like integrative and conjugal elements (ICEs).27

Furthermore, we analysed the regions surrounding the ampC 
genes. The genetic organization of blaCMY-2 and its variants was 
highly conserved. All the isolates carried the transposon-like 
element ISEcp1 (ISEcp1|ΔISEcp1-blaCMY-2-bla-sugE), as documented 
in previous reports.9,16,17 A well-conserved structure was 
also found in all isolates carrying blaACC-1 genes (ΔISEcp1-
blaACC-1-gdha). However, although IS26 is commonly related to 
the transmission of ACC-1 enzymes (and normally appears 
within ISEcp1),18,19 none of our isolates harboured this insertion 
sequence. The genetic organization of blaDHA1 was more 
variable. Mobilization of DHA-1 enzymes has been associated with
1. **bla**\textsubscript{CMY-2} and variants

1.1 **pNF4656-like**

\[ \text{a-b (amplified)} \]
\[ \text{ISEcp1} \]
\[ \text{bla}_{\text{CMY-2-like}} \]
\[ \text{blc} \]
\[ \text{sugE} \]

\[ \text{a-d (amplified; 2164 bp)} \]

1.2 **\Delta IS\textsubscript{Ecp1} 5’ end**

\[ \text{a-b (did not amplify)} \]
\[ \text{ISEcp1} \]
\[ \text{bla}_{\text{CMY-2-like}} \]
\[ \text{blc} \]
\[ \text{sugE} \]

\[ \text{a-d (did not amplify)} \]

1.3 **\Delta IS\textsubscript{Ecp1} 3’ end**

\[ \text{a-b (did not amplify)} \]
\[ \text{ISEcp1} \]
\[ \text{bla}_{\text{CMY-2-like}} \]
\[ \text{blc} \]
\[ \text{sugE} \]

\[ \text{a-d (amplified; 1584 bp)} \]

2. **bla\textsubscript{DHA-1}**

2.1 **Structure I (pTN60013-like)**

\[ \text{orfs-2} \]
\[ \text{bla}_{\text{DHA-1-like}} \]
\[ \text{ampR} \]
\[ \text{qacE}\Delta1\text{-sul1} \]
\[ \text{orf-5} \]

\[ \text{IS26} \]

\[ \text{DHA-1 (28)} \]

2.2 **Structure II (pT498-like)**

\[ \text{IS26} \]
\[ \text{orfs-2} \]
\[ \text{bla}_{\text{DHA-1-like}} \]
\[ \text{ampR} \]
\[ \text{qacE}\Delta1\text{-sul1} \]

\[ \text{DHA-1 (1)} \]

2.3 **Structure III**

\[ \text{orfs-2} \]
\[ \text{bla}_{\text{DHA-1-like}} \]
\[ \text{ampR} \]
\[ \text{qacE}\Delta1\text{-sul1} \]

\[ \text{DHA-1 (1)} \]

3. **bla\textsubscript{ACC-1}**

3.1 **Salmonella Bareilly 60.50-like**

\[ 5’\Delta IS\textsubscript{Ecp1} \]
\[ \text{bla}_{\text{ACC-1}} \]
\[ \text{gdha} \]

\[ 1 \text{ kb} \]

\[ \text{ACC-1 (3)} \]

**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 IS\textsubscript{Ecp1} truncated at the 5’ end. (1.3) 26.2\% of the isolates showed IS\textsubscript{Ecp1} 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 bla\textsubscript{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 sul1 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 sul1 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.

Acknowledgements
We would like to express our sincere thanks to Dr Llagostera (Dep. Microbiologia Molecular, UAB, Barcelona) for providing us with the E. coli HB101 (UA6190) strain and C. Newey for reviewing the English.

Funding
This study was partially supported by the Ministry of Health and Consumer Affairs, Instituto de Salud Carlos III-Feder, the Spanish Network for Research in Infectious Diseases (REIPI/RD06/0008/0013 and RD06/0008/1012), BFU2008-00995/BMC (Spanish Ministry of Education) and the European Union Seventh Framework Programme under grant agreement number 241476 (PAR project). A. A. was partially funded by the I Plan Regional of I Spain (catalan) and the European Union Seventh Framework Programme under grant agreement number 1590–3. M. P. G.-B. is the recipient of a JAE contract from Consejo Superior de Investigaciones Científicas (CSIC).

Transparency declarations
None to declare.

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

References
22 González-Sanz R, Herrera-León S, de la Fuente M et al. Emergence of extended-spectrum β-lactamases and AmpC-type β-lactamases in...


