Mobile genetic elements related to the diffusion of plasmid-mediated AmpC β-lactamases or carbapenemases from *Enterobacteriaceae*: findings from a multicenter study in Spain

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Running title: Genetic context of plasmid-mediated AmpC β-lactamases or carbapenemases in *Enterobacteriaceae*

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ABSTRACT

We examined the genetic context of 74 acquired *ampC* genes and 17 carbapenemase genes from 85 out of 640 *Enterobacteriaceae* isolates collected in 2009. Using S1-PFGE and Southern hybridization, 37 out of 74 bla_{AmpC} genes were located on large plasmids of different sizes belonging to six Inc groups. We used sequencing and PCR mapping to investigate the regions flanking the acquired *ampC* genes. The $bla_{CMY\text{-}2like}$ genes were associated with IS*Ecp1*, the surrounding bla_{DHA} genes were similar to *Klebsiella pneumoniae* plasmid pTN60013 associated with IS*26* and the *psp* and *sap* operons, and $bla_{ACC\text{-}1}$ genes were associated with IS*26* elements inserted into IS*Ecp1*. All the carbapenemase genes ($bla_{VIM\text{-}1}$, two $bla_{IMP\text{-}22}$ and $bla_{IMP\text{-}28}$) were located in class 1 integrons. Therefore, although plasmids are the main cause of the rapid dissemination of *ampC* genes among *Enterobacteriaceae*, we need to be aware that other mobile genetic elements, such as insertion sequences, transposons or integrons, can be involved in the mobilization of these genes of chromosomal origin. Additionally, three new integrons are described in this study (In846 to In848).

INTRODUCTION

β-lactam resistance in *Enterobacteriaceae* due to acquired Amp-C β-lactamases (pAmpC) or carbapenemases represents an emerging and increasing problem that limits therapeutic options. pAmpC confer resistance to most β-lactams, except cefepime and carbapenems, whereas carbapenemases, including class A, B and D, can confer resistance to most β-lactams, including carbapenems. The number of *Enterobacteriaceae* carrying these enzymes is lower than ESBLs-producing isolates, but it has increased over the last few years, particularly the pAmpC CMY-2 and DHA, and carbapenemases NDM, VIM, IMP and OXA-48 types (1, 2).

Both families of enzymes (pAmpC and carbapenemases) are normally codified in plasmids, and their genes are associated with mobile genetic elements (MGE) such as insertion sequences, transposon-like elements and class 1 integrons. All these MGE can transfer these genes into mobilizable and conjugative plasmids and subsequently disseminate them into many bacterial species that naturally lack these genes (3-5).

As previously described (3)**,** 100,132 *Enterobacteriaceae* isolates were collected from February to July 2009 from 35 Spanish hospitals. Among them, we found a total of 674 *Enterobacteriaceae* with acquired *amp*C and/or carbapenemase genes. The enzyme types found were: CMY-2-like (74.3%), followed by DHA (17.8%), ACC (1.5%), FOX (0.6%), VIM (4.3%) and IMP (1.5%) (3). Although a great genetic diversity among pAmpC-producing strains was observed, some clonal relationships were established between these isolates, mainly in carbapenemase-producing strains (3).

This study aimed to describe the plasmid families and the surrounding regions involved in the dissemination of a great diversity of acquired *amp*C and metallo-β-lactamases genes in *Enterobacteriaceae* isolates lacking inducible chromosomal AmpC enzymes.

MATERIAL AND METHODS

Clinical isolates. To characterize the plasmids and flanking regions implicated in the expansion of these genes, we selected 85 strains from the collection cited above (3). The selection was made on the basis of prevalence, and strains that produced new enzymes were also included.

PCR-based replicon typing. PCR-based Inc/rep typing (PBRT) was used to identify the major Inc groups of the plasmids present (4,6).

Plasmid profiles and Southern blot analysis. Plasmid analysis was carried out by DNA linearization with the S1 enzyme followed by pulsed-field gel electrophoresis (PFGE), as

previously described (7). Plasmid sizes were estimated using Fingerprinting II Informatix™ software (Bio-rad)(7). A PCR DIG Probe synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to obtain *bla*$_{AmpC}$ or Inc probes for hybridization of the S1-PFGE blots. These probes were labelled with the commercial kit (DIG high prime DNA labelling and detection starter kit II (Roche Diagnostics GmbH, Mannheim, Germany).

The chromosomal location of *bla* genes was analysed by digesting the genomic DNA with the ICeuI enzyme, followed by PFGE and hybridization blots, as described above.

Genetic environment characterization of acquired *ampC* and carbapenemase genes. The genetic context was investigated by exploring the regions surrounding acquired AmpC and carbapenemase genes frequently reported in the literature (8-14), employing PCR and sequencing with previously described primers. Additionally, primers designed in accordance with accessible DNA sequences in the GenBank (AY581207, AJ870924, Y11068, AJ971345 and EF577408) were used to ascertain the presence of genes linked to the acquired *bla*$_{ampC}$ and carbapenemase genes (Table S1). Sequencing reactions were performed with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA), and sequences were analyzed on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). The resulting sequences were then compared with those available at the GenBank (www.ncbi.nih.gov/BLAST).

RESULTS

Among the 85 selected strains (66 pAmpC-producing, 13 IMP/VIM-producing, 4 producing both enzymes and 2 strains that produced two pAmpC), we characterized the plasmids and flanking regions of 91 genes, 74 *bla*$_{ampC}$ and 17 *bla*$_{IMP/VIM}$ genes (Table 1).

The studied *bla*$_{ampC}$ genes included 40 *bla*$_{CMY-2-like}$, 22 *bla*$_{DHA}$, 8 *bla*$_{ACC}$ and 4 *bla*$_{FOX}$, while the metallo-β-lactamase genes included 14 *bla*$_{VIM-1}$, 2 *bla*$_{IMP-22}$ and 1 *bla*$_{IMP-28}$.

Plasmid characterization. Analysis by S1-PFGE and Southern hybridization allowed us to determine the plasmid size for 74.7% (68/91) of the studied genes, leaving 23 genes (25.3%) with a possible chromosomal location (positive hybridization in ICeuI-PFGE). Nevertheless, we were able to describe the incompatibility group by PCR-based replicon (PBRT-PCR) among these 68 plasmidic genes in only 41 cases (60.3%).

We found that 39 out of 74 *bla*$_{\text{AmpC}}$ genes (50%) were located in large plasmids of different sizes belonging to eight Inc groups, including: A/C, FIB, FIIA, I1, K, HI2, N and U. The most representative, present alone or together with other replicons, were: I1 (n=18, one also with FIB), K (n=7; two also with FIB), A/C (n=6), FIIA (2), HI2 (2) and N (2) (Table 1). In 13 out of 74 *bla*$_{\text{AmpC}}$ genes (17.5%), the plasmid replicon was not identified (in 10 cases the PBRT-PCR was positive for different replicons, but their hybridization bands did not match the *bla*$_{\text{AmpC}}$ band, and in 3 cases the PBRT-PCR was negative). Finally, in 22 cases (29.7%) a possible chromosomal location of these genes was confirmed by ICeuI-PFGE.

The plasmids carrying *bla*$_{\text{CMY-2-like}}$ genes belonged to the following Inc groups: I1 [16/40 (37.5%): sizes ranged from 43.7 to 145.5 kb), K (7/40 (17.5%: sizes ranged from 48.5 to 105.1 kb), and A/C [5/40 (12.5%): sizes ranged from 177.5 to 300.7 kb). In three cases the FIB plasmid was also found to be associated with one IncI1 or two IncK plasmids (Table 1).

Seven out of 22 plasmids carrying *bla*$_{\text{DHA}}$ genes were characterized, with the following Inc groups being found: I1 (9%: sizes 77.6 and 87.3 kb), FIIA (9%: size 76.6 and 218 kb), HI2 (9%: size 291 kb) and A/C (4.5%: 203.7 kb). The remaining 15 cases were not resolved, in 11 cases because no incompatibility group probes hybridized and in four cases because the PBRT-PCR was negative.

Only two of the eight plasmids carrying *bla*$_{\text{ACC-1}}$ were identified and they belonged to the IncN group, with sizes varying from 32.5 to 80 kb. *bla*$_{\text{FOX-3}}$ genes were found in plasmids of

72.5 and 80.5 kb, both of the IncU group, and the *bla*$_{FOX-8}$ gene (15) was probably located on the chromosome.

Finally, the sizes of the fourteen plasmids carrying the *bla*$_{VIM-1}$ gene ranged from 48 to 72.5 kb and one of them belonged to the IncU group; *bla*$_{IMP-22}$ genes were in plasmids of 485 kb with an unidentified Inc group. The *bla*$_{IMP-28}$ gene was probably located on the chromosome, as previously described (16).

Detection of the flanking regions of acquired *ampC* and metallo-β-lactamase genes.

The variable genetic environments detected for the most prevalent enzymes (CMY-2-like, DHA, ACC and metallo-β-lactamase genes) are shown in Figures 1-3.

The analysis of the genetic environment revealed that *bla*$_{CMY-2-like}$ genes (*bla*$_{CMY-2}$, *bla*$_{CMY-4}$, *bla*$_{CMY-7}$, *bla*$_{CMY-27}$, *bla*$_{CMY-48}$, *bla*$_{CMY-54/57}$, *bla*$_{CMY-59}$ and *bla*$_{CMY-60}$) were associated with IS*Ecp1*, responsible for the transfer of the *bla*$_{CMY-2-like}$-*blc*-*sugE* region from the chromosome of *Citrobacter freundii* to plasmids (8). In our study, 16 strains contained the IS*Ecp1* and *blc*-*sugE*-*ecnR* upstream and downstream of *bla*$_{CMY-2-like}$ genes, respectively. However, truncation of IS*Ecp1*(ΔIS*Ecp1*) was observed in 8 strains: four at the 3'end and four at the 5'end. In the former, primers described to explore this region (IS*Ecp1*/CMY2Ri) (Table S1) amplified a product of 1,560 bp instead of the expected 2,160 bp. In the latter, the amplicons were not obtained using IS*Ecp1*/CMY2Ri primers and we required a new pair of primers (TnpA1L/CMY2Ri). Finally, twelve strains did not contain IS*Ecp1* upstream, and in six strains the region downstream of the *bla*$_{CMY-2like}$ gene could not be amplified by PCR with the primers used. Only in two strains, with complete *bla*$_{CMY-2-like}$ gene, the genetic environment was unknown (Figure 1).

The surrounding regions of *bla*$_{DHA}$ genes (*bla*$_{DHA-1}$, *bla*$_{DHA-6}$ and *bla*$_{DHA-7}$) were similar to those previously described in *Klebsiella pneumoniae* plasmid pTN60013 (AJ971345) (5), although a certain variability was detected, in accordance with the literature data (11,17). This

variability mainly concerned the absence or presence of *sapB*, *sapA* and *sdr* genes (Figure 2). The quinolone resistance determinant *qnrB4* and additional *aadA1* (streptomycin and spectinomycin resistance) genes were detected in most of the strains. This linkage between bla_{DHA-1} and *qnrB4* genes has been previously described in isolates of *K. pneumoniae* (11).

In the environment of the bla_{ACC} gene, IS*Ecp1* and *gdhA* gene were detected upstream and downstream, respectively. In all cases, IS*Ecp1* was truncated in the 5'(13). Six out of eight bla_{ACC} genes showed two IS*26* copies in the same orientation; one of these strains contained a truncated 5' *gdh*A and one contained a *tnpR* gene of Tn5393 upstream of the *gdhA* gene.

Four bla_{FOX} genes, bla_{FOX-3} (n=2) and bla_{FOX-8} (n=2), were located in a class I integron, at the 5' of the integrase *intI1*, and several attempts to identify the 3 'end by PCR were unsuccessful.

All the metallo-β-lactamase genes (14 bla_{VIM-1}, 2 bla_{IMP-22} and 1 bla_{IMP-28}) were located in class 1 integrons. In this study, we detected five different structures harbouring bla_{VIM} genes (Figure 3), with *In*846 (GenBank accession number KC417378), *In*847 (KC417379) and *In*848 (KC417377) being described for the first time.

DISCUSSION

We have characterized the genetic context of the largest available collection of acquired AmpC β-lactamases and metallo-β-lactamases in *Enterobacteriaceae* lacking inducible chromosomal AmpC enzymes, recovered during 2009 from 35 Spanish hospitals (3).

Several authors have related the spread of different acquired AmpC genes with the expansion of plasmids of certain incompatibility groups (1, 2, 4,5,7,18-21). In this context, the bla_{CMY-2} gene is associated with plasmids of I1, A/C, and K incompatibility groups (7, 18-21). Our results match these data, but differences were found in the percentage of each incompatibility group. In a previous study (7), carried out during 1999-2007, A/C was the most predominant incompatibility group found (33%) among plasmids carrying bla_{CMY-2}, followed by I1 (23%)

and K (10%). In this study (with strains isolated in 2009), the most prevalent incompatibility group was I1 (40%), followed by K (17.5%) and A/C (12.5%). The fact that different Inc plasmids have been found to carry the same resistance gene is an indication of a successful and widespread distribution; moreover, this variability contributes to the genetic environment of these genes (20). The IncA/C and IncI1 are considered to be epidemic plasmids, because they are found in different countries, in bacteria of diverse origin and carrying a range of resistance mechanisms (4). IncA/C plasmids have been described carrying ESBLs TEM-type or VEB, as well as NDM-1 carbapenemase. On the other hand, IncI1 has an efficient conjugative system that could also contribute to the dissemination of different resistance mechanisms, such as ESBLs CTX-M-type and TEM-type (4).

The genetic environment of $bla_{\text{CMY-2}}$ and its variants was highly conserved. 60% of isolates carried the transposon-like elements IS*Ecp1*(IS*Ecp1*/ΔIS*Ecp1*-bla$_{\text{CMY}}$-blc-sugE), as documented in previous reports (8,10,18). As these $bla_{\text{CMY-2}}$-derived bla_{CMY} genes differ from one other by only a few nucleotide substitutions, it is possible that these differences could have evolved within the same Inc plasmid (I1) (20). The genes, $bla_{\text{CMY-55}}$ and $bla_{\text{CMY-56}}$, were found in the A/C plasmid, and the $bla_{\text{CMY-54}}$ gene in a K plasmid, in this case cointegrated with FIB.

$bla_{\text{DHA-1}}$ genes were initially associated with IncFII plasmids (20), but recent studies link them with IncL/M plasmids and *qnrB* determinants (5,7). Among our DHA-producing strains, 19 showed the *qnrB4* determinant (data not shown) and none were associated with the IncL/M plasmids. In fact, we were only able to characterize the plasmid in seven cases (38.8%), with I2, FIIA and HI2 being the incompatibility groups found. The genetic organization of bla_{DHA} genes was more variable. Mobilization of this enzyme has been associated with IS*26* or class 1 integron-bearing IS*CR1* elements (11,18). Among bla_{DHA}-carrying isolates, 86% were associated with IS*26*.

In the literature, characterization of plasmids carrying *bla*$_{ACC-1}$ genes is scarce. In a previous study, a *bla*$_{ACC-1}$ gene in an *Escherichia coli* strain was found in an IncI1 plasmid (7), but other authors could not type it (21). Regarding the genetic context of *bla*$_{ACC-1}$ genes, an IS*Ecp1*element truncated at the 5'end with an IS*26* insertion sequence was found in all of our *bla*$_{ACC-1}$-carrying isolates, as described in previous reports (12,13).

bla$_{FOX}$ and all carbapenemases detected in this study, including previously undescribed structures (*bla*$_{FOX}$), were located in a class 1 integron, and present in the most recent IncU plasmids.

There is little data on the types of plasmids involved in the spread of metallo-β-lactamases. In the literature, *bla*$_{IMP}$ and *bla*$_{VIM}$ genes are described in plasmids of incompatibility groups I1, N, W and HI2 (4). In this study, the *bla*$_{IMP-22}$ gene was located in a 485kb plasmid of an uncharacterised incompatibility group, and two *bla*$_{VIM-1}$ genes were found in IncU plasmids, curiously both isolated from different species but in the same hospital. Finally, the location of the *bla*$_{IMP-28}$ gene seems to be chromosomal, as *bla*$_{IMP-28}$ positive hybridization was found in the PFGE-ICeuI membrane.

Accordingly, the high number of unidentified replicons could be associated with plasmids other than those tested or, alternatively, they could be associated with one of the tested plasmids, albeit with some genetic variability, as has been described for the carbapenemase NDM in plasmids with a variant of the IncN or IncHI1 groups (4).

In conclusion, although plasmids have proven to be one of the main causes of the rapid dissemination of *bla*$_{AmpC}$ and carbapenemase genes among bacteria, other MGE must play an important role in the increasing prevalence of these enzymes. Further studies, focused not only on plasmids but also on other MGE, such as insertion sequences, transposons or ICEs, are needed to gain a better understanding of the complex process involved in the dissemination of antibiotic resistance genes worldwide.

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Figure legend

Figure 1. The genetic environment of *bla*$_{\text{CMY-2like}}$ and *bla*$_{\text{ACC}}$ genes, which are represented by solid black arrows, while the surrounding genes are represented by white arrows. The discontinued lines represent the areas that could not be amplified by PCR. Eight isolates indicate truncated versions of IS*Ecp1* at 3' (pattern A.1) and 5' end (pattern A.2). Truncated genes are represented by disrupted arrows. One isolate (pattern C) carrying the ACC gene showed an identical structure to AJ870924. In one isolate (pattern D), IS*26* was truncated by a *tnpR* gene of insertion Tn5393. The primers used for PCR amplification are also shown.

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Figure 2. The genetic environment of the *bla*$_{\text{DHA}}$ gene, which is represented by solid black arrows, while the surrounding genes are represented by white arrows. Continuous lines indicate an absence of the corresponding DNA fragments.

Figure 3. Structure of *bla*$_{\text{VIM-1}}$, *bla*$_{\text{IMP-22}}$ and *bla*$_{\text{IMP-28}}$ genes carrying the integrons described in this work. Carbapenemase genes are represented by solid black arrows and the surrounding genes are represented by white arrows The locations of the primers used for PCR amplification are also shown.

317 **Table 1**. Flanking regions and plasmid families associated with acquired AmpC and
318 carbapenemases in *Enterobacteriaceae*

***bla* genes**	**Flanking region**[a]	**Plasmid size (kb)**[b]	**Replicon**	**Microorganism (n)**
bla_{ACC-1}	ACC-1(B)	64.5	N	*K. pneumoniae* (1); *E. coli* (1)
		Chromosomal		*E. coli* (1); *P. mirabilis* (1)
		80	Unidentified	*K. pneumoniae* (1)
	ACC-1(C)	32.5	Unidentified	*P. mirabilis* (1)
	ACC-1(D)	48.5	Unidentified	*K. pneumoniae* (1)
bla_{ACC-1} + bla_{FOX-3}	ACC-1(A) + FOX (A)	64.5 + 80.5	Unidentified + U	*E. coli* (1)
bla_{CMY-2}	CMY-2(A)	177.5 to 300.7	A/C	*K. pneumoniae* (2); *P. mirabilis* (1)
		43.7 to 83.1	I1	*C. koseri* (1); *E. coli* (4)
		69.3	I1+ FIB	*E. coli* (1)
		48.5 to 83.1	K	*E. coli* (3); *K.pneumoniae* (1)
		Chromosomal		*E. coli* (1); *K. oxytoca* (1)
	CMY-2(B)	55.4	K	*E. coli* (1)
		76.2	I1	*E. coli* (1)
	CMY-2(C)	145.5	I1	*K. pneumoniae* (1)
		Chromosomal		*P. mirabilis* (3); *K. pneumoniae* (1); *P. penneri* (1)
	CMY-2(D)	83.1 to 97.6	I1	*E. coli* (1); *K. pneumoniae* (1)
bla_{CMY-4}	CMY-2(A)	105.1	K + FIB	*E. coli* (1)
		Chromosomal		*E. coli* (1)
	CMY-2(B)	80.8	I1	*E. coli* (1)
	CMY-2(C)	88.9	I1	*K. pneumoniae* (1)
bla_{CMY-7}	CMY-2(A)	80.8	I1	*E. coli* (1)
bla_{CMY-27}	CMY-2(A)	113.2	I1	*E. coli* (2)
bla_{CMY-43}	CMY-2(A)	Chromosomal		*E. coli* (1)
bla_{CMY-48}	CMY-2(B)	Chromosomal		*E. coli* (1)
bla_{CMY-54}	CMY-2(C)	105.1	K + FIB	*E. coli* (1)
bla_{CMY-55}	CMY-2(A)	282.9	A/C	*E. coli* (1)
bla_{CMY-56}	CMY-2(A)	299.1	A/C	*K. pneumoniae* (1)
bla_{CMY-57}	CMY-2(C)	97.0	I1	*E. coli* (1)
bla_{CMY-2} + bla_{DHA-1}	CMY-2(A) + DHA-1(G)	Chromosomal + 218	FIIA	*K. pneumoniae* (1)
bla_{CMY-2} + bla_{VIM-1}	CMY-2(C) + VIM-1(B)	Chromosomal + 48	Unidentified	*P. mirabilis* (1)
bla_{DHA-1}	DHA-1(A)	72.8 to 87.3	Unidentified	*C. koseri* (1); *E. coli* (1); *K. pneumoniae* (2)
		203.7	A/C	*K. pneumoniae* (1)
		Chromosomal		*C. koseri* (1); *E. cloacae* (1)
	DHA-1(B)	72.8	Unidentified	*K. oxytoca* (2)

		77.6	I1	*E. coli* (1)
	DHA-1(C)	Chromosomal		*P. mirabilis* (3)
	DHA-1(D)	72.8	Unidentified	*K. oxytoca* (1)
	DHA-1(E)	72.8	Unidentified	*Salmonella* spp. (1)
	DHA-1(F)	87.3	Unidentified	*E. coli (1)*
	DHA-1(H)	Chromosomal		*E. coli (1)*
bla_{DHA-6}	DHA-1(G)	87.3	I1	*E. coli* (1)
bla_{DHA-1} + bla_{VIM-1}	DHA-1(B) + VIM-1 (D) (*In*846)	76.6 kb + Chromosomal	FIIA	*K. pneumoniae* (1)
bla_{DHA-7} + bla_{VIM-1}	DHA-1(D) + VIM-1(E) (*In*847)	310.4 kb + 48.5 kb	HI2 + Unidentified	*E. cloacae* (2)
bla_{FOX-3}	FOX(A)	72.5	U	*E. coli* (1)
bla_{FOX-8}	FOX(A)	Chromosomal		*E. coli* (2)
bla_{IMP-22}	IMP-22(A)	485.0	Unidentified	*K. pneumoniae* (2)
bla_{IMP-28}	IMP-28(A) (*In*767)	Chromosomal		*K. oxytoca* (1)
bla_{VIM-1}	VIM-1(A) (*In*488)	48.0	Unidentified	*K. oxytoca* (1)
	VIM-1(B) (*In*624)	48.0 and 66.2	Unidentified	*E. cloacae* (2); *K. oxytoca* (2); *K. pneumoniae* (2)
	VIM-1(C) (*In*846)	48.0 and 72.5	U	*K. pneumoniae* (1); *E. cloacae* (1)
	VIM-1(D) (*In*848)	48.0	Unidentified	*E. coli* (1)

a, between brackets the type of surrounding regions found for each *bla* gene. More detailed data in Figures 1-3.

b, the plasmid size was determined after the hybridisation procedure.

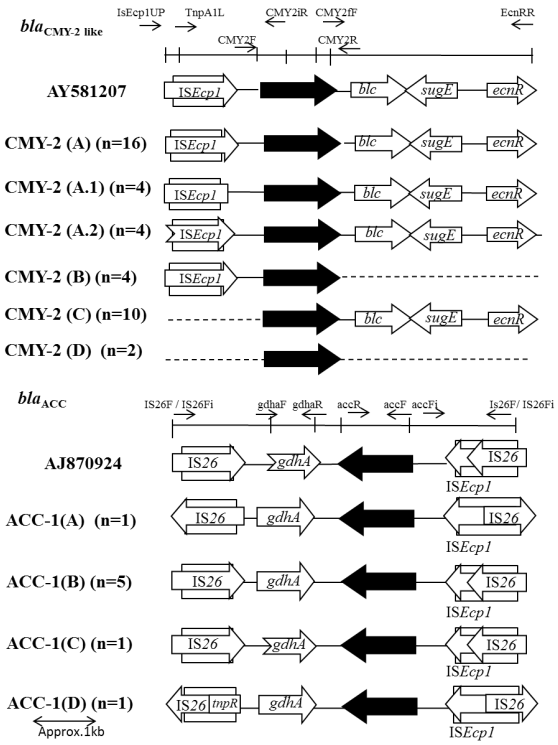

Figure 1. The genetic environment of *bla*$_{\text{CMY-2like}}$ and *bla*$_{\text{ACC}}$ genes, which are represented by solid black arrows, while the surrounding genes are represented by white arrows. The discontinued lines represent the areas that could not be amplified by PCR. Boxes corresponds to *TnpA* genes of each IS. Eight isolates indicate truncated versions of IS*Ecp1* at 3' (pattern A.1) and 5' end (pattern A.2). Truncated genes are represented by disrupted arrows. One isolate (pattern C) carrying the ACC gene showed an identical structure to AJ870924. In one isolate (pattern D), IS26 was truncated by a *tnpR* gene of insertion Tn5393. The primers used for PCR amplification are also shown.

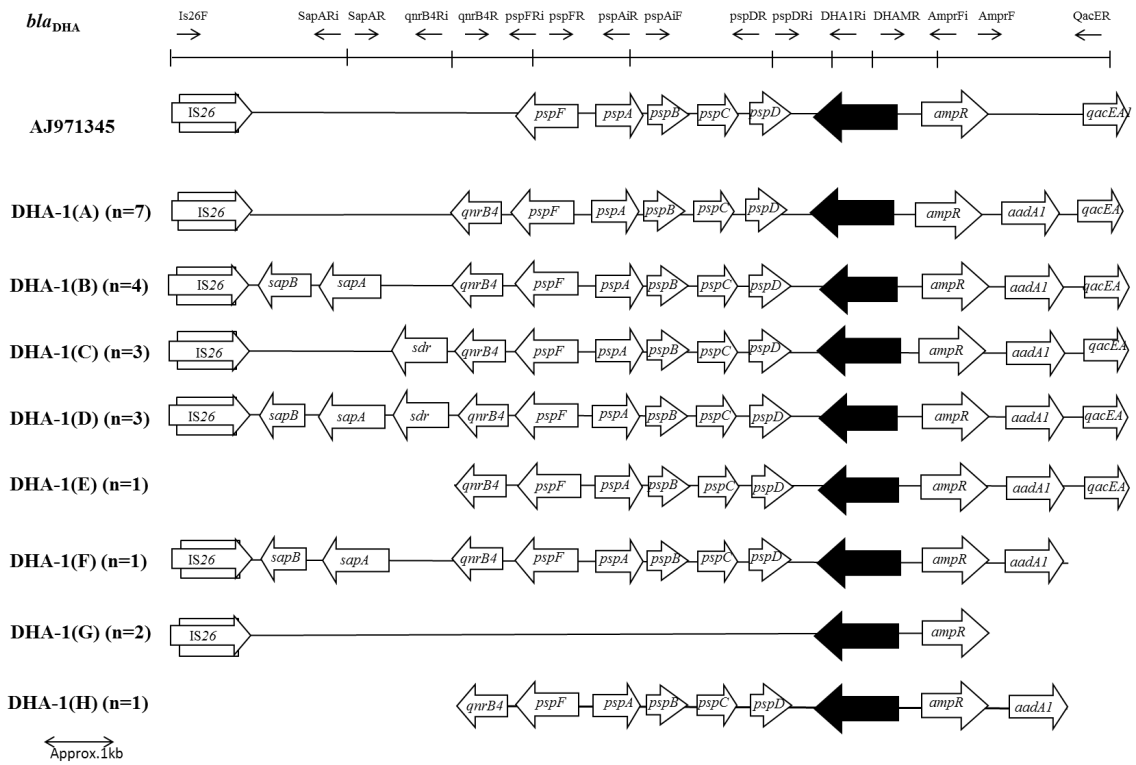

Figure 2. The genetic environment of the *bla*$_{DHA}$ gene, which is represented by solid black arrows, while the surrounding genes are represented by white arrows. Continuous lines indicate an absence of the corresponding DNA fragments. Boxes corresponds to *TnpA* genes of each IS.

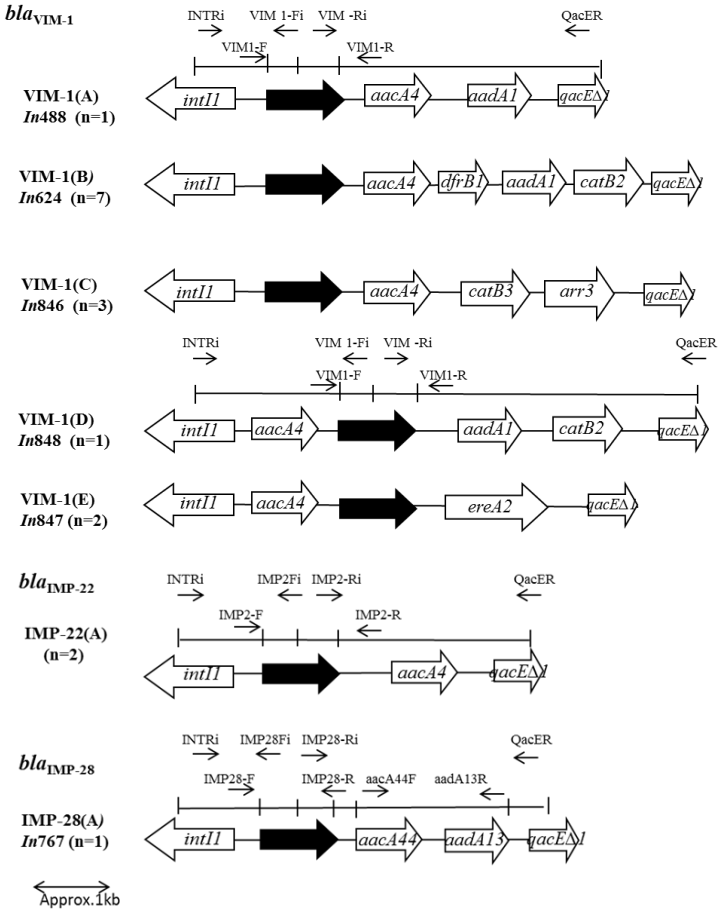

Figure 3. **3**. Structure of *bla*$_{VIM-1}$, *bla*$_{IMP-22}$ and *bla*$_{IMP-28}$ genes carrying the integrons described in this work. Carbapenemase genes are represented by solid black arrows and the surrounding genes are represented by white arrows The locations of the primers used for PCR amplification are also shown.