Analysis of *bla*$_{SHV-12}$-carrying *Escherichia coli* clones and plasmids from human, animal and food sources

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Short title: SHV-12-producing *E. coli* in humans, food and animals

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Synopsis

Objectives: This study aimed at characterizing 23 *Escherichia coli* isolates from various sources and their respective *bla*$_{SHV-12}$-carrying plasmids and sequencing one of these plasmids completely.

Methods: Isolates were typed by XbaI-PFGE, MLST and PCR-based phylotyping. Transformed *bla*$_{SHV-12}$-carrying plasmids were examined by replicon typing, S1-nuclease, conjugation, EcoRI-HindIII-BamHI digests and pMLST. Co-located resistance genes and integrons as well as the *bla*$_{SHV-12}$ genetic environment was analyzed by PCR and sequencing. One IncI1 plasmid was sequenced completely using HiSeq 2500 and gap closure by PCRs and Sanger sequencing.

Results: Among the 23 SHV-12-positive *E. coli*, some isolates from different sources showed the same characteristics: ST23/phylogroup A (human, dog, livestock), ST57/D (wild bird, chicken meat) and ST117/D (chicken meat, chicken). All *bla*$_{SHV-12}$ genes were horizontally transferable via 30-120 kb plasmids of incompatibility groups IncI1 (n=17), IncK (n=3), IncF (n=1), IncX3 (n=1) and a non-typeable plasmid. IncK plasmids, indistinguishable in size and restriction patterns, were found in isolates from different sources (ST57/D, meat; ST131/B2, meat; ST57/B1, dog). The IncI1-*bla*$_{SHV-12}$-carrying plasmids were mostly assigned to pST26 and pST3. Three plasmids showed novel pSTs (pST214, pST215). The majority of the IncI1 transformants exhibited resistance to β-lactams, chloramphenicol and streptomycin (in relation with a class 1 integron containing *estX-psp-aadA2-cmlA1-aadA1-qacI* gene cassette array), and to tetracycline. A novel *bla*$_{SHV-12}$ environment was detected and whole plasmid sequencing revealed a Tn*21*-derived-*bla*$_{SHV12}$-ΔTn*1721* resistance complex.

Conclusions: The results of this study suggest that the dissemination of *bla*$_{\text{SHV-12}}$ genes occurs by vertical (clonal) and horizontal transfer, the latter mainly mediated through IncI1 multidrug-resistance plasmids.

Introduction

The WHO has defined third- and fourth-generation cephalosporins as critically important antimicrobial agents in human medicine.[1] In Gram-negative bacteria, resistance to these antimicrobials has become a major health problem associated with the production of ESBLs such as those of TEM, SHV and CTX-M families. The presence of ESBL-producing *E. coli* has been widely reported not only in humans but also in food,[2] pets,[3] livestock,[4,5] and even wildlife.[6]

The CTX-M family is currently the most prevalent worldwide, but other ESBLs, such as SHV-12, remain important among pathogens causing nosocomial and community-acquired infections in many Southern European and Asian countries and have also been reported in *E. coli* isolated from livestock and wild birds.[4,6-10] Furthermore, SHV-12 was reported as the most prevalent enzyme detected in ESBL-producing Enterobacteriaceae from retail chicken meat and poultry in both Germany and Spain. [11,12]

Although several studies have examined mobile elements carrying $bla_{\text{CTX-M}}$ genes,[13,14] fewer data are available for the $bla_{\text{SHV-12}}$ gene. Thus, the aim of this study was to characterize a collection of $bla_{\text{SHV-12}}$-positive *E. coli* from different sources and geographical origins and their corresponding $bla_{\text{SHV-12}}$-carrying plasmids in order to gain insight into the presence and dissemination of this ESBL gene. Furthermore, we determined the complete sequence of a plasmid harboring the $bla_{\text{SHV-12}}$ gene in addition to several other resistance genes.

Materials and Methods

Bacterial collection, susceptibility testing and clonal characterization of $bla_{\text{SHV-12}}$-positive isolates

Twenty-three *bla*$_{SHV-12}$-positive *E. coli* from different sources and origins were analysed. Source of the Spanish isolates: A) Wild birds (n=4; starling, cuckoo, two storks); cloacal samples collected in the Aragón Reference Centre of wild-life recovering (La Alfranca, 2014).[6] B) Dogs (n=3); faeces of healthy dogs from different kennels (Logroño, 2009). C) Chicken meat samples (n=4) collected from different supermarkets (Logroño, 2011). D) Chickens (n=5); faeces of chickens from different slaughtherhouses (n=4) and a liver sample from a diseased animal (n=1) (Spain, 2003).[10] E) Humans (n=3); faecal samples of patients admitted to a Spanish hospital (June-July 2008). Source of the German isolates: tissue samples of diseased livestock birds (n=4; duck, turkey, two chickens); raised on different farms, collected by the German national resistance monitoring program (GE*RM*-Vet) (2010-2011).

All isolates were tested for susceptibility to ampicillin, amoxicillin/clavulanate, ceftazidime, ceftriaxone, cefoxitin, imipenem, nalidixic acid, ciprofloxacin, gentamicin, amikacin, tobramycin, streptomycin, chloramphenicol, compound sulphonamides, trimethoprim/sulfamethoxazole and tetracycline by disc diffusion according to the CLSI criteria.[15] ESBL production was verified by double-disc synergy test. *E. coli* ATCC 25922 served as control strain.

Carriage of the *bla*$_{SHV-12}$ gene was confirmed by PCR and sequencing.[16] Genetic diversity of the *bla*$_{SHV-12}$-positive isolates was analyzed using PCR-based phylotyping, MLST and XbaI-macrorestriction followed by PFGE.[17,18] A dendrogram, for the analysis of the XbaI-PFGE patterns, was generated using GelJ version 1.3 (UPGMA algorithm; Dice coefficient; 1% tolerance).[19]

Transfer and characterization of bla*$_{SHV-12}$*-carrying plasmids

Plasmids were transferred by conjugation and electrotransformation using the sodium azide-resistant *E. coli* J53 strain and electro-competent *E. coli* TOP10 as recipient cells, respectively.[20] Transconjugants and transformants were selected on Luria-Bertani agar supplemented with ceftazidime (1 mg/L) and sodium azide (200 mg/L) or with ceftazidime (1 mg/L), respectively.

Plasmids were characterized by PCR-based replicon typing, S1 nuclease digestion followed by PFGE and restriction fragment length polymorphism using the EcoRI, HindIII or BamHI endonucleases. [13,21] IncI1 plasmids were subtyped by plasmid MLST (pMLST).[22]

Antimicrobial resistance genes, integrons and bla*$_{\text{SHV-12}}$ *genetic environment

Genes associated with resistance to β-lactams (*bla*$_{\text{OXA}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$), aminoglycosides [*aac(3)-I, aac(3)-II, aac(3)-III, aac(3)-IV*, *strA*, *strB*], phenicols (*cmlA, floR, catB3*), quinolones (*qnrA, qnrB, qnrC, qnrD, qnrS, aac(6´)-Ib-cr, qepA, oqxA-oqxB*), sulphonamides (*sul1*, *sul2*, *sul3*) and tetracycline [*tet*(A-E)] were tested by PCR in all original isolates and *bla*$_{\text{SHV-12}}$-positive transformants.[23,24]

The presence of *intI1* and *intI2* genes, the variable region of the integrons and the genetic structure of their 3'-Conserved Segments (3'-CS) were determined by PCR and sequencing.[25,26] The variable region of the class 1 integron carried by *E. coli* C526 was annotated and submitted to the GenBank database (KU317749).

To elucidate the *bla*$_{\text{SHV-12}}$ genetic environment, a PCR strategy was carried out using previously reported primers.[27,28] To characterize the uncommon downstream region of the *bla*$_{\text{SHV-12}}$ gene in *E. coli* isolate 101689, a newly designed primer was used (DEOR_ge1: 5'-AGGGTACCGCTTTCCCAATC-3'). Its design was based on the draft sequence of the *bla*$_{\text{SHV-12}}$-carrying plasmid (pCAZ460, *E. coli* 101689) (data not shown).

Sequencing of bla$_{SHV-12}$*-carrying plasmids*

Plasmid sequencing of two *bla*$_{SHV-12}$-carrying plasmids pCAZ590 (*E. coli* 111918, from a chicken) and pCAZ460 (*E. coli* 101689, broiler origin) was performed using a HiSeq 2500, which produced 150-bp paired-end reads (Berry Genomics Company, Beijing, China). A draft assembly of the sequences was conducted using the CLC Genomics Workbench 5 (CLC Bio, Aarhus, Denmark); the assembly algorithm works by using de Bruijn graphs. The gap closure was performed by PCR and Sanger sequencing for pCAZ590. The draft sequence of pCAZ460 was used for the characterization of the *bla*$_{SHV-12}$ genetic environment and the incompatibility group.

A functional annotation of pCAZ590 was done using the RAST Prokaryotic Genome Annotation Server which was manually curated using the following bioinformatics tools: Artemis software, IS finder (www-is.biotoul.fr) and Swiss-Prot database (http://www.uniprot.org). EMBOSS Needle alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) was used for sequence comparison. Circular map of the plasmid was made using DNAPlotter.[29,30,31]

The *bla*$_{SHV-12}$ genetic environment of *E. coli* 101689 and the full-length sequence of plasmid pCAZ590 (*E. coli* 111918) were deposited in the EMBL database under accession numbers LT621755 and LT669764, respectively.

Results

Molecular typing of SHV-12-positive E. coli isolates

Table 1 shows the molecular diversity of the *bla*$_{SHV-12}$-positive *E. coli* collection. The 23 *bla*$_{SHV-12}$-carrying isolates displayed thirteen clones (ST/phylogroup), with ST23/A

(n=5), ST57/D (n=3), ST453/B1 (n=3), ST117/D (n=2) and ST405/D (n=2) as the most common ones. Three isolates from different wild bird species belonged to ST453 and were indistinguishable by XbaI-PFGE (pattern A).[6] Same was true for two ST23/A isolates from different dogs (pattern B) and two poultry isolates (pattern C). ST405 isolates from human origin were closely related (patterns E-E1) (Figure S1).

Some *bla*$_{\text{SHV-12}}$-positive *E. coli* isolates obtained from different sources shared the same characteristics: ST23/A (human, dog, duck, turkey), ST57/D (wild bird, chicken meat) and ST117/D (chicken meat, chicken). Additionally, isolates belonging to ST57 (wild bird, chicken meat, dog) showed closely related XbaI-PFGE patterns (D, D1, D2, D3) (Figure S1).

Transformation, conjugal transfer of ESBL-encoding genes and plasmid characterization

At least two replicon types were detected in each of the 23 *bla*$_{\text{SHV-12}}$-positive *E. coli*, with IncI1, IncFIB and IncF being the most common ones. All *bla*$_{\text{SHV-12}}$ genes were located on 30-120 kb plasmids of the incompatibility groups IncI1 (n=17), IncK (n=3), IncF (n=1) and non-typeable plasmids (n=2) and were transferable by transformation. Using the draft sequence one of these non-typeable plasmids, pCAZ460 (*E. coli* 101689), was assessed as an IncX3 plasmid by the PlasmidFinder server.[32] The ST131/B2 *E. coli* isolate harbored two ESBL genes located on different plasmids: *bla*$_{\text{SHV-12}}$ was detected on a 75-kb IncK plasmid (Table 2) and *bla*$_{\text{CTX-M-1}}$ on a 100-kb IncI1 plasmid (data not shown).

Among the 17 IncI1 *bla*$_{\text{SHV-12}}$-carrying plasmids, three of them showed novel plasmid STs (pSTs): one plasmid carried a transversion in *ardA* (pST214) and two other plasmids a novel allele combination (pST215) (Table 2). The three *E. coli* isolates from wild birds belonging to ST453/B1 carried closely related IncI1 plasmids

(indistinguishable restriction patterns) (Figure S2). The IncI1/pST26 plasmids harbored by ST23/A isolates from dogs (n=2) and the IncI1/pST215 plasmids carried by ST405/D isolates from humans (n=2) had the same size and resistance genotype, and showed related EcoRI, HindIII and BamHI restriction patterns.

IncK plasmids were found in isolates from different sources (ST57/D, chicken meat; ST131/B2, chicken meat; ST57/B1, dog), but showed equal sizes and EcoRI, HindIII or BamHI restriction patterns (Figure S2). These plasmids carried no additional resistance genes or integrons.

Conjugal transfer of the ESBL phenotype was demonstrated in all isolates except one (101908). IncK plasmids exhibited lower conjugation frequencies than IncI1 plasmids (10^{-5}-10^{-4} versus 10^{-4}-10^{-2}) (Table 2).

Co-located resistance genes and integrons

All original isolates of the studied collection showed multidrug-resistance phenotypes (resistance to antimicrobials of ≥ 3 different classes), except one (solely resistant to β-lactams and nalidixic acid) (Table 1). The German isolate 101689 (chicken, 2010) was the only one carrying a plasmid-mediated quinolone-resistance gene, specifically *qnrS1*. This gene was co-located with $bla_{\text{SHV-12}}$ on a 45 kb IncX3 plasmid. Although different genes encoding resistance to tetracycline [*tet*(A), *tet*(B)] and sulfonamides (*sul1*, *sul2*, *sul3*) were identified among the original isolates, we only found *tet*(B) and *sul3* genes (*sul1* in one isolate harboring a plasmid-borne class 1 integron) among $bla_{\text{SHV-12}}$-carrying transformants (Table 2). Overall, four resistance profiles were identified among $bla_{\text{SHV-12}}$-carrying transformants: β-lactams-tetracycline-chloramphenicol-streptomycin (10/23), β-lactams-chloramphenicol-streptomycin (5/23), β-lactams-tetracycline (2/23) and exclusively β-lactams (6/23).

Regarding integrons, 19/23 isolates carried class 1 and/or 2 integrons. Class 2 integrons were present in four ST57 isolates. Two different gene cassette (GC) arrays were detected: *dfrA1-sat2-aadA1* (n=3) and a recently described structure (IS*10-dfrA1-sat2-aadA1*).[33] Among *intI1*-positive isolates, three carried classic class 1 integrons containing different GC arrangements: *aadA1* (n=1), *dfrA1-aadA1* (n=1) and *qacG-aadA6-qacG* (n=1). The latter integron, reported as In*812* in INTEGRALL database, was co-located on the same plasmid as the *bla*$_{\text{SHV-12}}$ gene and was first reported in Enterobacteriaceae in this study. The 59-base element (*attC*) of the GC *aadA6* was truncated by the insertion of the second *qacG* gene. The coding region of both *qacG* cassettes was identical. Thirteen isolates and their transformants harbored a class 1 integron lacking the 3'-CS and containing a large array of GCs (*estX-psp-aadA2-cmlA1-aadA1-qacI*) (Table 1).

bla*$_{\text{SHV-12}}$ *genetic environment

Regarding the *bla*$_{\text{SHV-12}}$ flanking regions, IS*26* was located 73 bp upstream and the putative *deoR* transcriptional regulator gene 20 bp downstream of the *bla*$_{\text{SHV-12}}$ gene.

In isolate 101689, the *deoR* gene was truncated at position 698 (reverse direction) by the insertion of a 445-bp DNA segment preceding an IS*26* element. This fragment contained two ORFs, encoding a hypothetical protein and a putative ArsR family transcriptional regulator gene. The 17 nucleotides located at the 3'-end of this putative *arsR* gene overlapped with the IS*26* left inverted repeat (IRL) found downstream of the 445-bp segment (Figure S3).

Characteristics of the sequenced IncI1 plasmid pCAZ590

The completely sequenced plasmid pCAZ590 comprised 117,387 bp and displayed an average G+C content of 51.7% (Figure 1a). Replication, transfer and leading regions were highly similar to other IncI1 plasmids, with some insertions/deletions suggesting recombination between related plasmids. The entire region involved in conjugal transfer (*tra*/*trb* genes) was closely related (99.0% of identity) to that of the archetypal IncI1 plasmid R64 (accession no.AP005147). Larger portions of the backbone share high identity (99.0%) with plasmids PDM04 (NZ_CP013224.1), pSH1148_107 (NC_019123.1) and pSD107 (NC_019137.1) from different *Salmonella enterica* strains.

Plasmid pCAZ590 presented a large accessory module (26,728 bp) associated with antimicrobial resistance, located between the replication and the ColIb colicin immunity regions. This resistance module comprised a Tn*21*-derived transposon in which an atypical class 1 integron, the $bla_{\text{SHV-12}}$ gene and flanking elements (IS*26*-*deoR*) and a ΔTn*1721* transposon were inserted. It is located in pCAZ590 in the antisense orientation, but it is shown in (Figure 1a) and described in the text in the sense orientation to facilitate comparisons.

The Tn*21*-derived region carried the left and right Tn*21* terminal IRs, the genes involving its own transposition (*tnpA*, *tnpR*, *tnpM*), the terminal imperfect IRi of class 1 integron In*2* and the class 1 integrase *intI1* gene. However, almost the whole structure of integron In*2* was missing, solely a fragment of the *tniA* gene (615 bp) was identified. Instead of In*2*, an atypical integron was found, whose arrangement included the standard 5'-CS (*intI1* gene), the GC array *estX*-*psp*-*aadA2*-*cmlA1*-*aadA1*-*qacI* and the genetic platform IS*440*-*sul3*-*yqkA*-*yusZ*-*Δmef*(B)-IS*26*. The *mef*(B) gene, which encodes a macrolide-efflux protein, was found disrupted by the IS*26*. This atypical class 1 integron was detected in most of the isolates of our collection. The segment IS*26*-$bla_{\text{SHV-12}}$-*deoR* was followed by a ΔTn*1721*, encoding resistance to tetracycline. The ΔTn*1721* contained

the two characteristic regions of Tn*1721*, the first corresponding to the genes involved in the production of a putative chemotaxis protein (*orf1*) and transposition (*tnpR*, *tnpA*) was complete. However, the second region contained the tetracycline transcriptional regulator and resistance genes [*tetR, tet*(A)] and a *pecM*-like gene, but the truncated transposase (Δ*tnpA*) and the terminal inverted repeat IRRII were missing. From the mercury resistance module (*merRTPCAD*) typically located in Tn*21*, only a fragment of *merR* (120 bp) was found downstream of the ΔTn*1721*-Δ*tniA* (Figure 1b).

Discussion

This study focused on the investigation of the $bla_{\text{SHV-12}}$ gene, which codes for one of the most prevalent ESBLs. The $bla_{\text{SHV-12}}$-positive isolates were collected over different periods of time and from different sources and geographical areas. The repeated occurrence of distinct clones in different sources/years of isolation suggests the presence of potentially "epidemic" clones with relatively high stability over time, including the closely related resistance plasmids carried by them.

Some of the most common clones (ST23/A, ST57/D) detected in this study have been associated with ESBL phenotypes and are widely spread among different environments, including clinical settings.[34] A member of the epidemic ST131/B2 clone, associated with CTX-M production, was detected in one chicken meat sample harboring both $bla_{\text{SHV-12}}$ and $bla_{\text{CTX-M-1}}$ genes. According to the high prevalence of SHV-12 enzyme among non-ST131 *E. coli* in Spain, the occurrence of a horizontal $bla_{\text{SHV-12}}$ transfer event from local non-ST131 isolates to the epidemic ST131 clone has been suggested.[34] This hypothesis is supported by our findings, which confirm a high similarity between IncK

plasmids detected in isolates belonging to the ST131 and other STs, reinforcing the possibility of a horizontal transfer of this *bla*$_{SHV-12}$-carrying plasmid to the ST131 lineage.

The *bla*$_{SHV-12}$ gene was mainly associated with IncI1 plasmids. These plasmids seemed to be easily transferred by conjugation with high efficiency, which may explain their predominance in different ecosystems.[22,35] Although a considerable diversity was found, IncI1/pST3 and IncI1/pST26 appeared to be the dominant subtypes. In agreement with previous reports,[22,35] all the IncI1/pST3 plasmids harboring *bla*$_{SHV-12}$ were detected in poultry or poultry-derived meat suggesting a potential association between this variant and poultry. Conversely, *bla*$_{SHV-12}$-carrying IncI1/pST26 plasmids and related subtypes belonging to CC26 (like pST29), were associated with a wide host-range contributing to the spread of SHV-12 encoding genes among different environments and geographical areas. In fact, pST29 detected in wild birds isolates (2014) and the novel pST215, identified in two commensal *E. coli* isolates from humans (2008), may reflect a possible diversifying evolutionary process.

It is also remarkable that, in contrast to IncK-positive transformants, all susceptible to non-β-lactams antimicrobials, 14 *bla*$_{SHV-12}$-carrying IncI1 plasmids and one non-typeable plasmid showed a multidrug-resistance phenotype associated (i) with the presence of the same atypical class 1 integron containing *aadA2-cmlA1-aadA1* GCs, and (ii) frequently, with the co-location of the *tet*(A) gene. In particular, the structure of this atypical integron (*intI1-estX-psp-aadA2-cmlA1-aadA1-qacI*-IS*440-sul3*) appears to be identical to that found by other authors on *bla*$_{SHV-12}$-carrying IncI1 plasmids. It is usually embedded in Tn*21*-derived transposons and is globally distributed among Enterobacteriaceae from different environments.[36]

As some authors have suggested and as confirmed by the pCAZ590 plasmid sequence, the high prevalence of this atypical integron seems to be associated with its

downstream linkage to IS*26*, which constitutes the highly conserved upstream flanking region of the *bla*$_{SHV-12}$ gene (Figure 1b).[36,37] The presence of these genetic platforms plays an important role in the persistence of SHV-12-producing isolates due to their capability to promote the selection of these ESBL genes under the selective pressure imposed even by antimicrobial agents other than β-lactams.

Regarding genetic environments, it is noteworthy that 22/23 isolates showed an identical genetic structure flanking the *bla*$_{SHV-12}$ gene. However, the novel described downstream environment revealed the truncation of the putative *deoR* transcriptional regulator gene by a genetic structure containing two ORFs preceding an IS*26*. Such genetic structure has been shown to be truncating other genes in different regions of many plasmids [pYD626E (KJ933392) and pSRC119-A/C (KM670336)], revealing its high mobility potential. This may be due to the ability of IS*26* to mobilize neighboring genes by misidentifying short sequences as its alternative left-hand IR. The insertion of this genetic structure may have important implications due to the putative composite transposon formed, which could facilitate the exchange *en-bloc* of the ESBL gene (Figure S3).

As a final remark, the first described Portuguese *E. coli* isolate carrying an IS*10* within a class 2 integron[33] and the one identified in this study belonged in both cases to ST57, suggesting that this specific class 2 integron may be clonally disseminated.

Although the present study provides important insights into the understanding of the dynamics and the molecular background of *bla*$_{SHV-12}$-carrying *E. coli* isolates, future studies, using larger numbers of isolates, are needed to identify other potential epidemic clones/plasmids. Moreover, the large sampling period (2003-2014) may represent a drawback due to the rapid evolution of ESBL genes. However, based on our findings, it

seems unlikely that the molecular background of *bla*$_{SHV-12}$-carrying clones/plasmids has changed dramatically over the sampling period.

Overall, this study revealed that some SHV-12-producing *E. coli* isolates from different sources showed identical ST/PFGE profile or carried highly similar plasmids. These observations suggest that both clonal and plasmid transfer facilitates the spreading of *bla*$_{SHV-12}$ ESBL genes. Horizontal dissemination was mainly driven by IncI1 plasmids showing rather conserved co-located resistance genes.

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Transparency declarations

None to declare

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Table 1. Molecular typing, resistance phenotype, integrons and resistance genes of *bla*$_{SHV-12}$-positive *E. coli* isolates.

E. coli isolate (source[a], origin[b])	Year of isolation	ST (Clonal Complex)	Phylo-group	PFGE pattern	ESBL	Resistance phenotype to non-β-lactams[c]	Class 1 integron		Class 2 integron		Resistance genes (outside the integron)
							intI1/3'-CS[d]	Variable Region	*intI2*	Variable Region	
C7377 (W, Sp)	2014	57 (350)	D	D3	SHV-12	CHL-CIP-NAL-STR-SUL-SXT-TET	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	+	*dfrA1-sat2-aadA1*	*tet*(B)
C7385 (W, Sp)	2014	453 (86)	B1	A	SHV-12	CHL-CIP-GEN-NAL-STR-SUL-TET-TOB	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(A), *aac(3)-II*
C7394 (W, Sp)	2014	453 (86)	B1	A	SHV-12	CHL-CIP-GEN-NAL-STR-SUL-TET-TOB	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(A), *aac(3)-II*
C7401 (W, Sp)	2014	453 (86)	B1	A	SHV-12	CHL-CIP-GEN-NAL-STR-SUL-TET-TOB	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(A), *aac(3)-II*
C4746 (M, Sp)	2011	57 (350)	D	D2	SHV-12	CIP-NAL-STR-SUL-SXT-TET	-/-	-	+	*dfrA1-sat2-aadA1*	*tet*(B), *sul2*
C4748 (M, Sp)	2011	57 (350)	D	D1	SHV-12	CIP-NAL-STR-SUL-SXT-TET	-/-	-	+	IS*10-dfrA1-sat2-aadA1*	*tet*(B),*aadA, sul2*
Pn461 (M, Sp)	2011	117	D	H	SHV-12	CIP-NAL-SUL-SXT-TET	-/-	-	-	-	*tet*(A), *sul2*, *bla*$_{TEM-1}$
C4745 (M, Sp)	2011	131	B2	G	SHV-12, CTX-M-1	NAL-SUL-TET	-/-	-	-	-	*tet*(A), *sul2*
C2585 (D, Sp)	2009	23 (23)	A	B	SHV-12	CHL-NAL-STR-SUL-TET	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(A)
C2578 (D, Sp)	2009	23 (23)	A	B	SHV-12	CHL-NAL-STR-SUL-TET	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(A)
C2575 (D, Sp)	2009	57 (350)	B1	D	SHV-12	CIP-NAL-STR-SUL-SXT-TET	+/+	*aadA1*	+	*dfrA1-sat2-aadA1*	*tet*(A), *tet*(B), *sul2*
C1536 (H, Sp)	2008	23 (23)	A	F	SHV-12	CHL-CIP-NAL-STR-SUL-SXT-TET	(a) +/+ (b) +/-	*(a) dfrA1-aadA1* *(b) estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(A), *sul2*
C1537 (H, Sp)	2008	405 (450)	D	E	SHV-12	CHL-CIP-NAL-STR-SUL-TET	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(A), *tet*(B)
C1538 (H, Sp)	2008	405 (450)	D	E1	SHV-12	CHL-CIP-NAL-STR-SUL-TET	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(A),*tet*(B)
C353 (L, Sp)	2003	155 (155)	B1	H	SHV-12	CHL-STR-SUL	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	-
C515 (L, Sp)	2003	1564	A	I	SHV-12	CHL-STR-SUL-TET	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(A), *sul2*, *bla*$_{TEM-1}$
C508 (L, Sp)	2003	2001	D	J	SHV-12	CHL-NAL-STR-SUL-TET	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(B), *bla*$_{TEM-1}$
C526 (L, Sp)	2003	362	D	K	SHV-12	SUL-TET	+/+	*qacG-aadA6-qacG*	-	-	*tet*(A), *tet*(B), *sul2*
C537 (L, Sp)	2003	616 (155)	B1	L	SHV-12	SUL-TET	-/-	-	-	-	*tet*(A), *tet*(B), *sul3*
101689 (L, Ge)	2010	117	D	M	SHV-12	NAL	-/-	-	-	-	*qnrS1*
101908 (L, Ge)	2010	23 (23)	A	C	SHV-12	CHL-NAL-STR-SUL-TET	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(A)
101985 (L, Ge)	2010	23 (23)	A	C	SHV-12	CHL-NAL-STR-SUL-TET	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(A)
111918 (L, Ge)	2011	371 (350)	D	N	SHV-12	CHL-STR-SUL-TET	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-	-	*tet*(A), *sul1*

[a] W: wild bird; M: chicken meat; D: dog; H: human; L: livestock bird (poultry). [b]Sp: Spain; Ge: Germany. [c] CHL: chloramphenicol; CIP: ciprofloxacin; GEN: gentamicin; NAL: nalidixic acid; TOB: tobramycin; STR: streptomycin; SUL: compound sulphonamides; SXT: trimethoprim-sulfamethoxazole; TET: tetracycline. [d] Class 1 integrons displaying atypical 3'-Conserved Segment (CS) were identified with the negative result for the investigation of the usual structure of 3'-CS and they were associated to IS*440-sul3*. The positive results for the 3'-CS indicate the class 1 integrons displaying a usual 3'CS, which were, as expected, associated to *qacΔE1-sul1*.

Table 2. Characteristics of *bla*$_{SHV-12}$-carrying plasmids in the studied *E. coli* collection.

E. coli isolate (source[a], origin[b])	Year of isolation	ST/phylogroup	Replicon type	*bla*$_{SHV-12}$-carrying plasmid							Other co-located resistance genes
				Replicon type	IncI1 pMLST (ST/CC)	Size (kb)	Conjugation frequency	Genetic environment of *bla*$_{SHV-12}$	Class 1 integron		
									intI1/3'-CS	Variable Region	
C7377 (W, Sp)	2014	ST57/D	FIB, F, I1	I1	214	100	2.2 x 10^{-3}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-
C7385 (W, Sp)	2014	ST453/B1	FIB, F, I1	I1[c]	29 (26)	115	3.5 x 10^{-3}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	*tet*(A)
C7394 (W, Sp)	2014	ST453/B1	FIB, F, I1	I1[c]	29 (26)	115	5.2 x 10^{-3}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	*tet*(A)
C7401 (W, Sp)	2014	ST453/B1	FIB, F, I1	I1[c]	29 (26)	115	3.7 x 10^{-3}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	*tet*(A)
C4746 (M, Sp)	2011	ST57/D	B/O, FIB, F, I1	I1	3 (3)	90	5.6 x 10^{-3}	IS*26*-*bla*$_{SHV-12}$-*deoR*	-/-	-	-
C4748 (M, Sp)	2011	ST57/D	FIB, F, K	K[d]	-	75	1.9 x 10^{-4}	IS*26*-*bla*$_{SHV-12}$-*deoR*	-/-	-	-
Pn461(M, Sp)	2011	ST117/D	FIB, F, I1	I1	3 (3)	90	3.5 x 10^{-3}	IS*26*-*bla*$_{SHV-12}$-*deoR*	-/-	-	-
C4745 (M, Sp)	2011	ST131/B2	FIB, F, I1, K	K[d]	-	75	9.6 x 10^{-5}	IS*26*-*bla*$_{SHV-12}$-*deoR*	-/-	-	-
C2585 (D, Sp)	2009	ST23/A	FIB, F, I1	I1	26 (26)	110	3.1 x 10^{-3}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	*tet*(A)
C2578 (D, Sp)	2009	ST23/A	FIB, F, I1	I1	26 (26)	110	2.0 x 10^{-3}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	*tet*(A)
C2575 (D, Sp)	2009	ST57/B1	FIB, F, I1, K, P	K[d]	-	75	7.9 x 10^{-5}	IS*26*-*bla*$_{SHV-12}$-*deoR*	-/-	-	-
C1536 (H, Sp)	2008	ST23/A	FIB, F, I1	F	-	90	1.7 x 10^{-2}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	*tet*(A)
C1537 (H, Sp)	2008	ST405/D	FIA, F, I1	I1	215	110	7.4 x 10^{-4}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	*tet*(A)
C1538 (H, Sp)	2008	ST405/D	FIA, F, I1	I1	215	110	6.9 x 10^{-4}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	*tet*(A)
C353 (L, Sp)	2003	ST155/B1	F, I1	I1	178	110	8.7 x 10^{-3}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-
C515 (L, Sp)	2003	ST1564/A	A/C, FIB, F, I1	I1	3 (3)	100	8.3 x 10^{-3}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-
C508 (L, Sp)	2003	ST2001/D	FIB, F, I1	I1	3 (3)	105	2.0 x 10^{-3}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-
C526 (L, Sp)	2003	ST362/D	FIB, F, I1, Y	I1	26 (26)	110	1.5 x 10^{-2}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/+	*qacG-aadA6-qacG*	*tet*(A)
C537 (L, Sp)	2003	ST616/B1	FIA, FIB, F, I1, Y	I1	26 (26)	105	2.0 x 10^{-2}	IS*26*-*bla*$_{SHV-12}$-*deoR*	-/-	-	*tet*(A)
101689 (L, Ge)	2010	ST117/D	FIB, F, I1	X3	-	45	4.4 x 10^{-6}	IS*26*-bla$_{SHV-12}$-Δ*deoR*	-/-	-	*qnrS1*
101908 (L, Ge)	2010	ST23/A	FIB, F, I1	NT[e]	-	30	NC[f]	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	-
101985 (L, Ge)	2010	ST23/A	FIB, F, I1	I1	26 (26)	110	1.2 x 10^{-7}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	*tet*(A)
111918 (L, Ge)	2011	ST371/D	FIB, F, I1	I1	95	120	1.0 x 10^{-4}	IS*26*-*bla*$_{SHV-12}$-*deoR*	+/-	*estX-psp-aadA2-cmlA1-aadA1-qacI*	*tet*(A)

[a] W: wild bird; M: chicken meat; D: dog; H: human; L: livestock bird (poultry). [b]Sp: Spain; Ge: Germany. [c, d] These plasmids show indistinguishable patterns after EcoRI, HindIII or BamHI digestion. [e] NT: non-typeable. [f] NC: non-conjugative (under the tested conditions, please see the Materials and Methods section).

Figure Legends:

Fig. 1. (a) Circular map of plasmid pCAZ590 (accession number LT669764) and **(b)** linear illustration of the complex multidrug-resistance region of pCAZ590 and a comparative analysis of this region, Tn*21* (accession number AF071413) and T*1721* (accession number X61367). Some relevant genes are labeled. In **(a)** the second inner ring shows the fragments of truncated genes and the forward and reverse coding sequences are shown in the third and fourth inner rings, respectively. They are shown as arrows (the direction of transcription is indicated by the arrow heads) and are colored according to their function as shown in the legend. The names of functional regions are shown in the outer ring. The first inner ring shows a plot of the GC skew (yellow, above average; purple, below average). In order to facilitate comparisons, in **(b)** the sequence is shown according to the orientation described for Tn*21* and Tn*1721*, although in the pCAZ590 it is found in the opposite orientation. The coding reading frames are shown as arrows (the direction of transcription is indicated by the arrow heads) and are colored as described in (a). The insertion sequences are presented as boxes and the arrows within the boxes indicate the transposition genes. The vertical lines represent the inverted repeats (IR) of insertion sequences, transposons or integron In*2*.

Fig. 1a

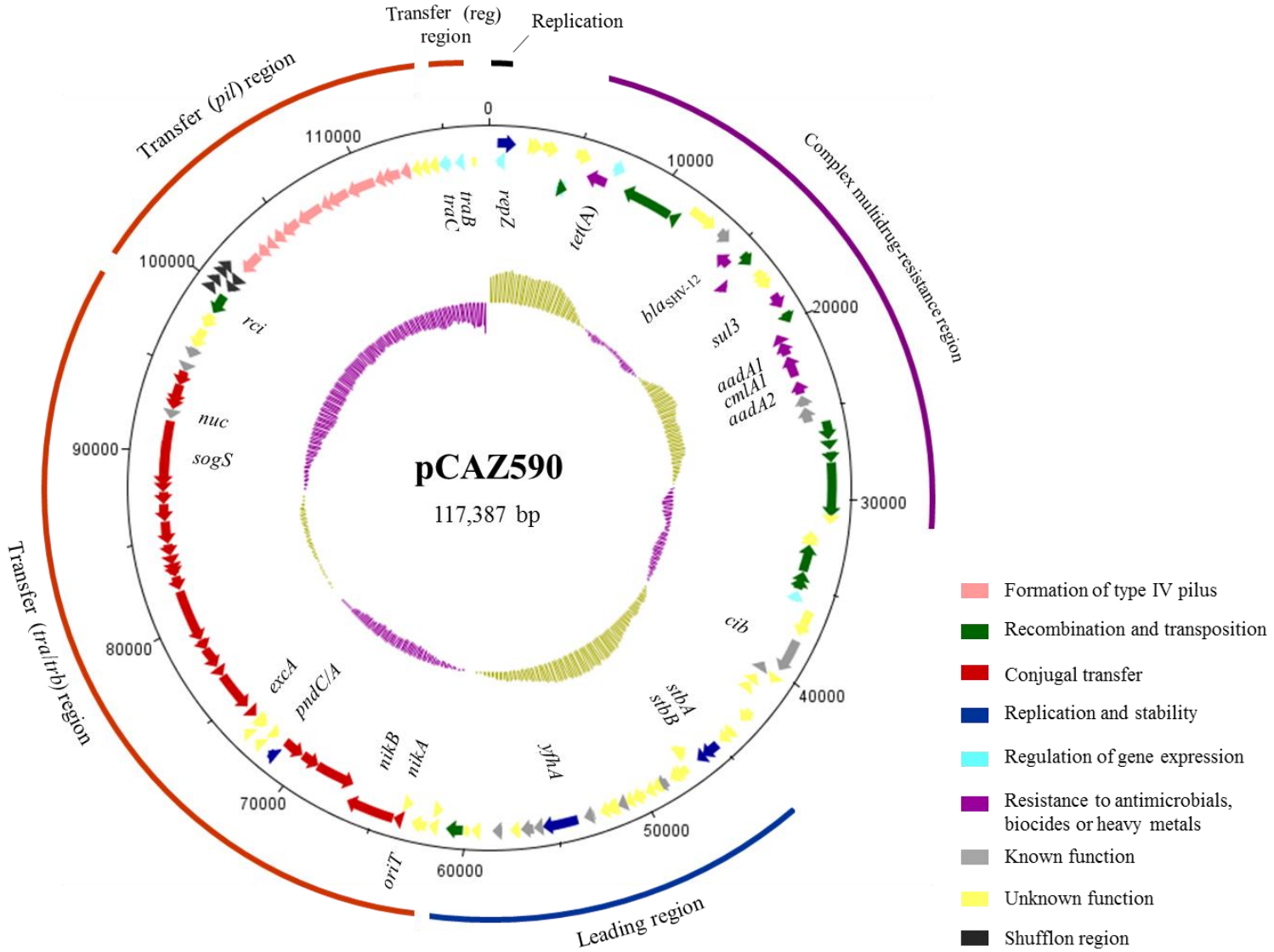
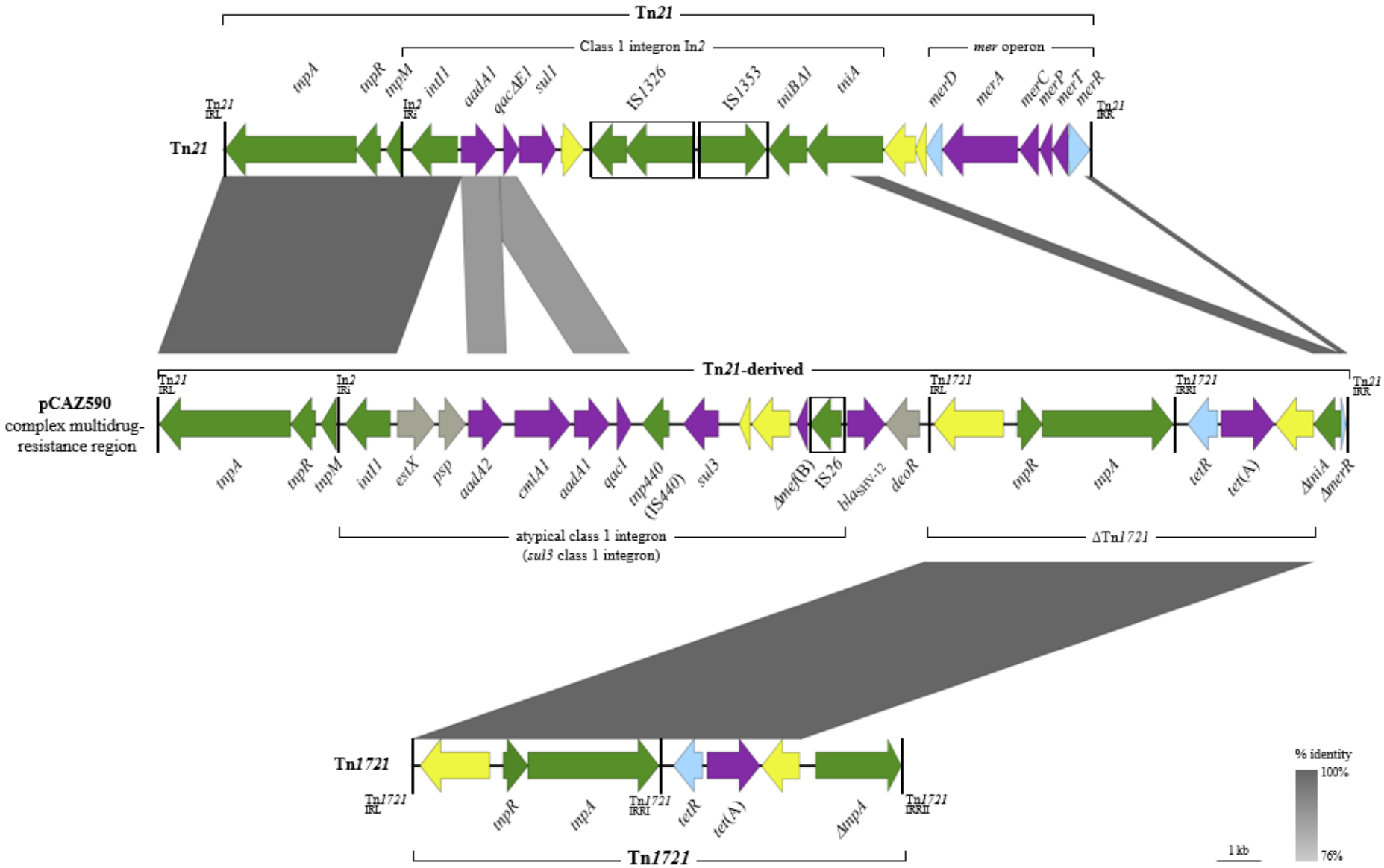

Transfer (reg) region
Replication
Transfer (pil) region
Complex multidrug-resistance region
Transfer (tra/trb) region
Leading region
pCAZ590
117,387 bp
0
10000
20000
30000
40000
50000
60000
70000
80000
90000
100000
110000
traB
traC
repZ
tet(A)
blaSHV-12
sul3
aadA1
cmlA1
aadA2
cib
stbA
stbB
yfhA
nikB
nikA
pndC/A
excA
nuc
sogS
rci
oriT
Formation of type IV pilus
Recombination and transposition
Conjugal transfer
Replication and stability
Regulation of gene expression
Resistance to antimicrobials, biocides or heavy metals
Known function
Unknown function
Shufflon region

Fig. 1b


Tn21
Class 1 integron In2
mer operon
tnpA
tnpR
tnpM
intI1
aadA1
qacΔE1
sul1
IS1326
IS1353
tniBΔ1
tniA
merD
merA
merC
merP
merT
merR
Tn21 IRL
In2 IRi
Tn21 IRR
Tn21
Tn21-derived
pCAZ590
complex multidrug-resistance region
Tn1721 IRL
Tn1721 IRRI
Tn21 IRR
tnpA
tnpR
tnpM
intI1
estX
psp
aadA2
cmlA1
aadA1
qacI
tnp440 (IS440)
sul3
Δmef(B)
IS26
blaSHV-12
deoR
tnpR
tnpA
tetR
tet(A)
ΔtniA
ΔmerR
atypical class 1 integron
(sul3 class 1 integron)
ΔTn1721
Tn1721
Tn1721 IRL
Tn1721 IRRI
Tn1721 IRRII
tnpR
tnpA
tetR
tet(A)
ΔtnpA
% identity
100%
76%
1 kb