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New TEM-Derived Extended-Spectrum β-Lactamase and Its Genomic Context in Plasmids from *Salmonella enterica* Serovar Derby Isolates from Uruguay

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A small (8.2-kb) ColE1 plasmid encoding TEM-144 (a new β-lactamase with a ceftazidimase profile) was sequenced by a gene-walking strategy. The *bla*$_{TEM}$ allele was carried on a Tn*2* element, disrupting a Rom protein gene. TEM-144 differs from TEM-1 by two mutations (R164C and E240K) and from the ceftazidime-hydrolyzing TEM-91 by one mutation (T182M).

The TEM-derived extended-spectrum β-lactamases (ESBLs) include more than 100 variants (11) originated by one to four amino acidic modifications relative to TEM-1 or TEM-2. Modifications of a few residues have been described to be responsible for TEM-derived ESBL activity, namely, Glu-104, Arg-164, Ala-237, Gly-238, and Glu-240 (17). Interestingly, *bla*$_{TEM}$ genes are frequently embedded in transposable elements such as Tn*2* inserted in wide-host-range plasmids (12).

Two *Salmonella enterica* serovar Derby isolates with decreased susceptibility to ceftazidime were detected in a previous study of samples of poultry origin (6). An 8.2 kb-plasmid (pSD1 and pST12) was obtained from isolates SD1 and ST12. Both plasmids showed identical restriction patterns when digested with PstI, displaying three fragments of about 5, 3, and 0.7 kb (data not shown), and could be transferred by transformation (24) to *Escherichia coli* strain CAG12177 [F$^-$ λ$^-$ *zej-298*::Tn*10*(Tet^r) *gyrA261*(Nal^r) *rph-1*] (*E. coli* Genetic Stock Center). MICs were determined as specified by the CLSI (formerly NCCLS) (18). Both transformants CAG12177/pSD1 and CAG12177/pST12 had identical antibiotic susceptibility profiles, which were also similar to those of isolates SD1 and ST12 (Table 1). The MICs of ceftriaxone and ceftazidime, with and without clavulanic acid, suggested that they produced enzymes hydrolyzing ceftazidime more efficiently than ceftriaxone (Table 1). Transformants remained fully susceptible to the carbapenems and to other families of antibiotics such as aminoglycosides, tetracyclines, quinolones, and sulfonamides.

Crude extracts of both original and transformant strains displayed, after isoelectrofocusing (21), a single β-lactam-hydrolyzing band at an apparent pI of 5.6 when revealed with 0.5 mM nitrocefin (data not shown).

PCRs (25) with specific *bla*$_{TEM}$ primers were positive when plasmid DNA (both from the original poultry isolates as well as from the transformants) was used as a template but yielded negative results with primers specific for *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{PER-2}$, and *bla*$_{OXA}$ genes (for primer descriptions, see Table 2). Sequence analysis of PCR products revealed the presence of a *bla*$_{TEM}$ allele encoding a new TEM-derived enzyme, showing two amino acid differences compared to TEM-1 (R164C and E240K). These modifications occurred by two nucleotide substitutions (in boldface type): **C**GT→**T**GT at position 692 and **G**AG→**A**AG at position 917, respectively, as described previously by Ambler et al. (1). Only one difference could be detected compared with TEM-91 (13), namely, T182M (A**T**G→A**C**G). The amino acidic sequence of mature TEM-144 had a predicted pI of 5.6, consistent with the value previously observed by isoelectric focusing.

TEM-144 has two mutations known to be involved in the extension of substrate specificity of TEM-type enzymes and could explain the resistance profile conferred by this enzyme. A substitution at position 164 (Arg→Cys) could break a salt bridge formed between residues 164 and 179 in the Ω-loop, allowing the entrance of ceftazidime molecules in the oxyanionic pocket, as previously revealed by Majiduddin and Palzkill (16). It should be noted that of 40 TEM-derived ESBLs with R164 modifications, only two (TEM-87 [20] and TEM-91 [13]) contain an R164C substitution (11). On the other hand, substitutions of E240 were always E240K (11). However, only the following three TEM-derived ESBLs have only two modifications, including one at position 240 and another additional change: TEM-10 (R164S) (22), TEM-28 (R164H) (7), and TEM-71 (G238S) (23).

The low-level resistance to oxyimino cephalosporins except ceftazidime (Table 1) could hamper laboratory detection of this ESBL if the latter drug is not used in detection tests.

Eleven primers were designed during a gene-walking strategy (Table 2) to determine the full sequence of the 8.2-kb

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TABLE 1. Antibiotic susceptibility of *S. enterica* serovar Derby SD1 and ST12 isolates and transformants

Antibiotic[c]	MIC (μg/ml)[a]			
	Serovar Derby		CAG12177	CAG12177/ $bla_{TEM-144}$[b]
	SD1	ST12		
Ampicillin	>256	>256	4	>256
Ampicillin-CLA	4	4	4	8
Piperacillin	32	64	1	32
Piperacillin-tazobactam	2	2	1	1
Cephalothin	16	16	16	16
Cefuroxime	8	8	2	4
Cefoxitin	4	4	1	4
Ceftazidime	32–64	64	<0.125	32
Ceftazidime-CLA	1	1	<0.125	0.125
Ceftriaxone	0.5	2	0.064	0.25
Ceftriaxone-CLA	0.125	0.125	0.064	0.064
Cefepime	0.5–2	1–4	0.5	0.5–2
Imipenem	0.25	0.25	0.25	0.25
Meropenem	<0.06	<0.06	<0.06	<0.06

[a] Values obtained by the agar dilution method.
[b] The same data were obtained with transformants CAG12177/pST12 and CAG12177/pSD1.
[c] Clavulanic acid (CLA) and tazobactam concentrations were always 4 μg/ml.

plasmids. Sequences were assembled using NCBI tools, resulting in a complete circular sequence (Fig. 1), and were identical for the two plasmids. The sequence contains three different regions encoding antimicrobial resistance, replication, and mobilization functions, respectively.

The antimicrobial resistance region zone includes three open reading frames (ORFs) corresponding to the *tnpA*, *tnpR*, and bla_{TEM} coding sequences. The bla_{TEM} promoter was recognized upstream of bla_{TEM} (regions $^{-35}$TTCAAA and $^{-10}$G ACAAT) and belongs to the weak promoter type *P3* with the following three conserved nucleotides: C32, G162, and G175 (14). This antibiotic resistance gene was incorporated into a transposable element identical to Tn*2*∗, which was described previously by Partridge and Hall (19). A replicative region involved in the regulation of replication of ColE1-like plasmids (9) was found upstream of Tn*2*∗. This region encodes RNA II (promotes plasmid replication) and RNA I (antisense down-regulation element) and contains a putative oriV. A fourth element, the gene *rom* (the only coding sequence in the replication origin), was truncated by Tn*2* insertion (see below). Downstream of Tn*2*∗, a horizontal plasmid mobilization system was detected, and this transmissibility region contains a *mobA* gene and oriT elements (5). An additional region is a multimer resolution site, an important mechanism for the regeneration of monomers in high-copy-number plasmids. The rest of the plasmid included four ORFs without any known function.

After excluding the Tn*2* region from the plasmid, the queries for alignment yielded only three significant matches corresponding to three closely related plasmids, namely, pOSAK1 (gi4589704), p4821 (gi3152962) (10), and NTP16 (gi9507429) (8). pOSAK1 and p4821 are small plasmids without transposable elements, but NTP16 presents a complete Tn*4352* that confers kanamycin resistance and a truncated Tn*2* that confers ampicillin resistance. Interestingly, in pST12/pSD1 and in NTP16, a Tn*2* element is inserted, interrupting a Rom protein gene, including the same "TTCTT" target sequence (8). The presence of Tn*2* disrupting the gene *rom* could favor a higher plasmid copy number by inactivating the replication inhibitor (3). This fact may lead to at least two linked consequences: an increase in the copy number of bla_{TEM} and consequently a larger amount of enzyme, which might have an impact on the level of resistance (15). Likewise, a larger number of bla_{TEM} copies per bacterial cell could increase the chances of generating new variants of β-lactamases with better hydrolytic capabilities.

Even if *Salmonella enterica* serovar Derby is not one of the serovars most frequently associated with human diarrhea, the fact that SD1 and ST12 were detected in the human food chain (hen eggs) points to the possibility of accession to the human digestive tract and spreading in the clinical setting.

TABLE 2. Primers used for PCR and sequencing

Primer no.	Primer name	Sequence (5′→3′)	Target DNA	Reference, source, or EMBL accession no.
1	OTR3	ATGAGTATTCAACAT TTC CG	bla_{TEM}	2
2	OTR4	CCAATGCTTAATCAG TGA GG	bla_{TEM}	2
3	TEM.DOWN	TCGTAGTTATCTACACGAAGG	bla_{TEM}	This work
4	TEM-UP	TATTGTCTCATGAGCGGATAC	bla_{TEM}	This work
5	TnpA-UP	TCACGCTGGCAGAACTGGTGA	*tnp*A	This work
6	TnpA-UP2	CAGGTTGCTGCACGTCTTTGC	*tnp*A	This work
7	TnpA-UP3	CTTATGCAACATCGTATTATT	*tnp*A	This work
8	TnpA-DW	GATGCCATGAAAGCCGGAATA	*tnp*A	This work
9	TnpA-DW2	ACGAATCGATTCGTCCGGTGT	*tnp*A	This work
10	TnpA-DW3	ATTAATATGTCTATTAAATCG	*tnp*A	This work
11	MobA-DW	TATCGAAGGCAAGCGTGTCGG	*mob*A	This work
12	Bla1	TTAATGATGACTCAGAGCATT	$bla_{CTX-M-2}$	X92507
13	Bla2	GATACCTCGCTCCATTTATTGC	$bla_{CTX-M-2}$	X92507
14	blaOXA-2F1	CCT GCA TCG ACA TTC AAG ATA	bla_{OXA-2}	X03037
15	blaOXA-2R1	CTC AAC CCA TCC TAC CCA CCA	bla_{OXA-2}	X03037
16	blaSHV-2R1	ATT TCG CTC GGC CAT GCT CGC	bla_{SHV-2}	M59181
17	blaSHV-2F1	ATG ATC ACC ACC TTT AAA GTA	bla_{SHV-2}	M59181
18	PER-2F	TGTGTTTTCACCGCTTCTGCTCTG	bla_{PER-2}	4
19	PER-2R	AGCTCAAACTGATAAGCCGCTTG	bla_{PER-2}	4

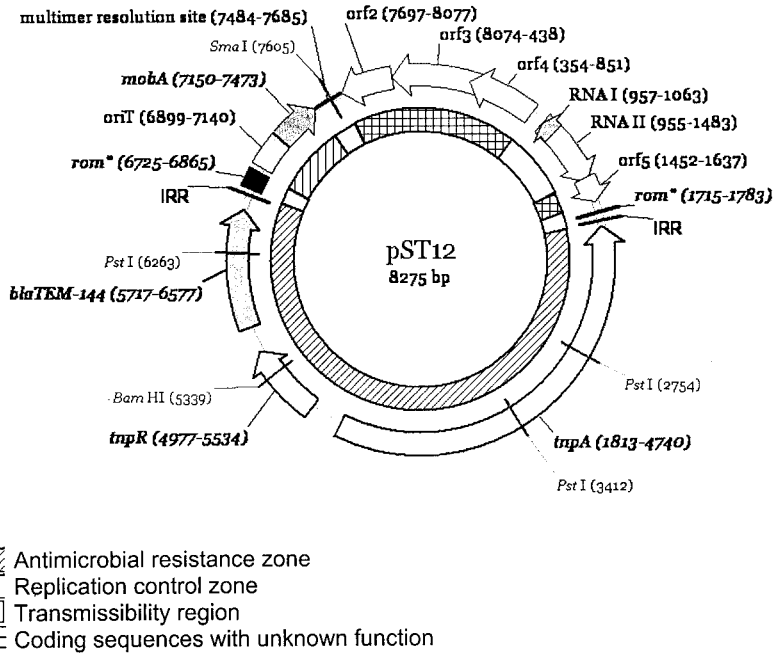

FIG. 1. Circular genetic map of pST12. The inner circle represents the three zones with a known function. The truncated Rom protein gene is depicted in black. ORFs are indicated by arrows pointing in the direction of transcription. Gene names and their coordinates are displayed in boldface type. Restriction endonucleases and their cutting sites are shown in regular type. IRR, inverted repeat region.

Nucleotide sequence accession numbers. The sequences of TEM-derived ESBLs were deposited into the EMBL database under accession numbers AM049399 and AM049400.

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