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Novel Plasmid-Encoded Ceftazidime-Hydrolyzing CTX-M-53 Extended-Spectrum β-Lactamase from *Salmonella enterica* Serotypes Westhampton and Senftenberg[▽]

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We describe the characterization of a novel CTX-M β-lactamase from *Salmonella enterica*. Four *S. enterica* isolates (three of serotype Westhampton and one of serotype Senftenberg) resistant to extended-spectrum cephalosporins (cefotaxime and ceftazidime) were recovered in 2004 from living cockles in three supermarkets located in distant geographic areas in France, which got their supplies from the same fishery. The isolates were found to produce a novel extended-spectrum β-lactamase (ESBL) belonging to the CTX-M-1 phylogenetic group and named CTX-M-53. The CTX-M-53 β-lactamase harbored the substitution Asp240Gly, like the CTX-M-15 enzyme, which is specifically implicated in a higher catalytic efficiency against ceftazidime. The $bla_{CTX\text{-}M\text{-}53}$ gene was located on a mobilizable 11-kb plasmid, pWES-1. The complete sequence of pWES-1 revealed the presence of a novel insertion sequence, IS*Sen2*, and an IS*26* element upstream and downstream of the $bla_{CTX\text{-}M\text{-}53}$ gene, respectively; however, transposition assays of the $bla_{CTX\text{-}M\text{-}53}$ gene were unsuccessful. IS*26* elements may have contributed to the acquisition of the $bla_{CTX\text{-}M\text{-}53}$ gene. Interestingly, the mobilization module of the pWES-1 plasmid was similar to that of quinolone resistance plasmids (carrying the *qnrS2* gene) from aquatic sources. Although belonging to two serotypes differentiated on the basis of the O-antigen structure (E1 or E4 groups), the isolates were found to be genetically indistinguishable by pulsed-field gel electrophoresis. Multilocus sequence typing showed that the isolates of serotype Westhampton had a sequence type, ST14, common among isolates of serotype Senftenberg. This is the first characterization of the CTX-M-53 ESBL, which represents an additional ceftazidime-hydrolyzing CTX-M enzyme.

Salmonella enterica is a frequent pathogen of animals and humans. Food-borne diseases caused by this species represent an important public health problem worldwide. Extended-spectrum cephalosporins (ESC) and fluoroquinolones are often used in the treatment of invasive cases of salmonellosis. However, the emergence of extended-spectrum β-lactamases (ESBLs) in *Enterobacteriaceae* is an increasing problem worldwide, compromising the utilization of these drugs in the treatment of complicated *Salmonella* infections. Moreover, there is an increasing number of reports of ESBL-producing *S. enterica* strains throughout the world (3, 36). These strains, isolated mostly in hospitalized patients, produced plasmid-mediated class A ESBLs belonging to the TEM, SHV, cefotaximase (CTX-M), or PER families (3).

CTX-M ESBLs are class A ESBLs that in general possess a higher level of hydrolytic activity against cefotaxime (and ceftriaxone) than against ceftazidime but that are inhibited by clavulanic acid, sulbactam, and tazobactam (5, 9, 26). On the basis of their amino acid sequences, the CTX-M enzymes have been classified into five major phylogenetic branches, namely the CTX-M-1, -2, -8, -9, and -25 groups (5, 9, 26; http://www.lahey.org/Studies/other.asp). CTX-M ESBLs are a rapidly growing group, which contains ESBLs encoded by more than 80 identified CTX-M genes (http://www.lahey.org/Studies/other.asp). In the genus *Salmonella*, 14 different CTX-M β-lactamases have been reported in several serotypes and over wide geographic areas (3, 5, 20, 21, 36–38). We report here the characterization of the novel CTX-M-53 ESBL in *S. enterica* serotypes Westhampton and Senftenberg in France. The *S. enterica* strains showing an ESBL phenotype were recovered from cockles from the Etel River (Morbihan, France) in August 2004. A molecular characterization of the β-lactamase gene was done by PCR, cloning, and sequencing. The genomic diversity of the isolates was determined by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The novel CTX-M enzyme was characterized by MIC determination of the β-lactams, isoelectric focusing (IEF), and kinetic parameters. The CTX-M-carrying plasmid was fully sequenced, and $bla_{CTX\text{-}M}$ mobilization experiments were performed.

MATERIALS AND METHODS

Bacterial strains and plasmids. The four *S. enterica* isolates were collected from cockles fished from the Etel River (Morbihan, France) and distributed in

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Phenotype, genotype, and/or characteristics[a]	Reference or source (year of isolation)
Strains		
S. enterica serotype Westhampton		
04CEB8118SAL	ESBL, $bla_{CTX\text{-}M\text{-}53}$	Living cockles, fishery, Morbihan, France[b] (2004)
04CEB8273SAL	ESBL, $bla_{CTX\text{-}M\text{-}53}$	Living cockles, supermarket, Puy-de-Dôme, France[b] (2004)
04CEB8276SAL	ESBL, $bla_{CTX\text{-}M\text{-}53}$	Living cockles, supermarket, Vienne, France[b] (2004)
S. enterica serotype Senftenberg		
04CEB8275SAL	ESBL, $bla_{CTX\text{-}M\text{-}53}$	Living cockles, supermarket, Vienne, France[b] (2004)
E. coli		
ATCC 25922	Control in disk diffusion method and in MIC determinations	CDC, Atlanta, GA
DH1	F$^-$ *endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1*	
DH5α	DH1 genotype and F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169*	
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 ara*Δ*139* Δ(*ara*, *leu*)*7697 galU galK-rpsL nupG tonA*	Invitrogen
HB101	*hsd20*(r_B^- m_B^-) *recA13 rpsL20*(Str^r) *leu proA2*	
Plasmids		
pBK-CMV phagemid	Neo^r Kan^r	Boehringer-Mannheim
pOX38-Neo^r	F derivative Tra$^+$ Neo^r, 58.2 kb	18
pGBG1	pBBR1 derivative Chl^r, 7.6 kb	35
pWES-1	11-kb natural plasmid from *S. enterica* 04CEB8273SAL electrotransformed in *E. coli* DH10B	This study
pCTX-M-53	1.5-kb PCR fragment from *S. enterica* 04CEB8273SAL that contained $bla_{CTX\text{-}M\text{-}53}$ in the XbaI-PstI sites of pBK-CMV	This study

[a] Str^r, streptomycin resistant; Neo^r, neomycin resistant; Kan^r, kanamycin resistant; Chl^r, chloramphenicol resistant.
[b] Département, French administrative subdivision.

three supermarkets across the country from August to September 2004. The detection of the *Salmonella* sp. was performed by using an enzyme-linked immunosorbent assay-based test, Transia Plate Salmonella (Raisio Diagnostics SAS, Lyon, France), and by the reference cultural method EN ISO 6579:2002. The *Salmonella* strains were identified using API 20E strips (bioMérieux, Marcy l'Etoile, France) and serotyped at the AFSSA laboratory (French Agency for Food Safety) on the basis of somatic O and phase 1 and phase 2 flagellar antigens by agglutination tests with antisera (Bio-Rad, Marnes la Coquette, France, and the WHO Collaborative Center for Reference and Research on *Salmonella*, Institut Pasteur, Paris, France) as specified by the White-Kauffmann-Le Minor scheme (19). Table 1 shows details of all the bacterial strains and plasmids used in this study.

Antimicrobial susceptibility testing. Antibiotic susceptibility was determined by the disk diffusion method with 32 antimicrobial drugs (Bio-Rad), as previously described (38). The MICs of ceftriaxone and ceftazidime were determined by Etest (AB Biodisk, Solna, Sweden). The ESBL phenotype was detected by using the ESBL detection Etest strips (AB Biodisk) and the double-disk synergy method (16). The isolates were categorized as susceptible, intermediate, or resistant according to Antibiogram Committee of the French Society for Microbiology (CA-SFM) clinical breakpoints (http://www.sfm.asso.fr/nouv/general.php?pa=2). The clinical breakpoints used for ceftriaxone and ceftazidime are slightly different from those determined by the Clinical and Laboratory Standards Institute (CLSI); susceptible strains were thus defined by a MIC of ≤4 μg/ml (CLSI, ≤8 μg/ml) and resistant strains by a MIC of >32 μg/ml (CLSI, ≥64 μg/ml for ceftriaxone and ≥32 μg/ml for ceftazidime).

Escherichia coli ATCC 25922 was used as a control for the disk diffusion method and for the MIC determinations.

PCR amplification of β-lactamase genes and sequence analysis. The total DNA of *S. enterica* isolates was extracted using the InstaGene matrix kit (Bio-Rad) in accordance with the manufacturer's recommendations. PCR amplifications of the bla_{TEM}, bla_{SHV}, $bla_{OXA\text{-}1}$ group, and $bla_{CTX\text{-}M}$ group genes were performed using TEM-F and TEM-R, SHV-F and SHV-R, OXA-1-F and OXA-1-R, CTX-M-F and CTX-M-R, or CTX-M-1-F and CTX-M3/M15-R primers, respectively, as described previously (37, 38). Sequencing was performed at Genome Express (Meylan, France). The nucleotide sequences and the deduced protein sequences were analyzed with EditSeq and Megalign software (Dnastar, Madison, WI). The BLASTN program of NCBI was used for database searches (http://www.ncbi.nlm.nih.gov/BLAST/).

PFGE. The genetic diversity of the *Salmonella* isolates was assessed by the PFGE of genomic DNA digested with XbaI (Roche, Mannheim, Germany), as described previously (22). The running conditions and the molecular size marker were as described in the standardized PulseNet protocol (34). BioNumerics 4.0 (Applied Maths, Sint-Martens-Latem, Belgium) was used for image normalization and the construction of similarity matrices. Bands were assigned manually. Clustering was carried out by the unweighted-pair group method with arithmetic averages based on the Dice similarity index, using a 1% optimization parameter and 1% band position tolerance.

MLST. The genomic DNA was prepared from a bacterial culture plate using the Wizard kit (Promega, Madison, WI). The seven genes for the MLST analysis, *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*, were amplified using the primers and PCR conditions described elsewhere (http://mlst.ucc.ie/mlst/dbs/Senterica/documents/primersEnterica_html). Both strands of purified amplicons were sequenced by the Genotyping of Pathogens and Public Health platform (PF8; Institut Pasteur) and nucleotide sequences obtained with BigDye version 3.1 chemistry (Applied Biosystems, Foster City, CA) on an ABI 3700 apparatus (Applied Biosystems). Alleles were assigned by comparing the sequences to those in the *Salmonella* MLST database hosted by University College Cork, Cork, Ireland.

ESBL resistance transfer and plasmid analysis. A resistance transfer experiment was carried out on liquid or solid media as described previously (37). *E. coli* DH1 resistant to nalidixic acid (Nal) was used as the recipient strain. Transconjugants were selected on Drigalski agar (Bio-Rad) supplemented with cefotaxime (2 μg/ml) and Nal (50 μg/ml). The electroporation of plasmid DNA, extracted by the QIAfilter Plasmid Midi kit (Qiagen), from S. *enterica* isolates to *E. coli* DH10B was performed using a GenePulser apparatus (Bio-Rad). The transformants were selected on Mueller-Hinton agar containing ceftazidime (4 μg/ml). Plasmid DNA was extracted from *E. coli* transformants by an alkaline lysis procedure (37) and subjected to 0.8% agarose gel electrophoresis. The molecular sizes of the plasmids were determined by reference to plasmids of known sizes, RP4 (54 kb) and pIP173 (126 kb), mixed with a supercoiled DNA

ladder (Invitrogen, Groningen, The Netherlands). Plasmid DNAs from the *E. coli* transformants were digested using the AvaI restriction enzyme (Promega) and compared by agarose gel electrophoresis, as previously described (37). The pWES-1 plasmid from *S. enterica* isolate 04CEB8273SAL was fully sequenced on both strands by directional genome walking using internal primers (at Genome Express). The nucleotide sequences and the deduced protein sequences were analyzed with EditSeq and Megalign software (Dnastar). The BLASTN program of NCBI was used for database searches.

Determination and cloning of the novel $bla_{CTX\text{-}M\text{-}53}$ gene. The sequences of the entire $bla_{CTX\text{-}M\text{-}53}$ gene and the surrounding DNA were obtained by directional genome walking of the pWES-1 plasmid. The $bla_{CTX\text{-}M\text{-}53}$ entire gene was PCR amplified by using primers CLON05-686F/PstI (5′-GGGCTGCAGGCTGGAG CCGCACCAGAGCCAAA-3′) and CLON05-686R/XbaI (5′-CCCTCTAGAAC GGAATGAGTTTCCCCATTCCG-3′) located outside the open reading frame (ORF) and comprising external endonuclease restriction sites (underlined). The amplification was performed on a 50-μl sample containing plasmidic DNA from pWES-1 (1 μl), primers (50 pmol each), deoxynucleoside triphosphates (100 μM each), DNA polymerase (1 U Isis proofreading DNA polymerase; Qbiogene, Illkirch, France) and its buffer, and dimethyl sulfoxide (10%). The cycling conditions included 10 min of denaturation at 94°C (1 cycle) and 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 1 min 30 s of polymerization at 72°C (35 cycles), followed by 10 min of extension at 72°C.

The amplified product (1,064 bp) was digested with PstI and XbaI (Roche) and cloned into PstI- and XbaI-digested phagemid pBK-CMV using T4 DNA ligase (Roche). The transformation of the recombinant plasmid was performed using heat shock transformation with DH5α-competent *E. coli* (Invitrogen). The transformants were selected on Mueller-Hinton agar containing cefotaxime (5 μg/ml). One *E. coli* transformant containing recombinant plasmid pCTX-M-53 with an insert having 100% nucleotide identity compared to the corresponding sequence of pWES-1 was selected for further studies.

IEF. IEF was performed with polyacrylamide gels containing ampholines with a pH range of 3.5 to 10 as previously described (6). The following β-lactamases of known isoelectric points (pIs; in parentheses) were used as standards: CTX-M-14 (7.9), CTX-M-1 (8.4), and CTX-M-15 (8.6).

β-Lactamase preparation. The CTX-M-producing *E. coli* DH5α(pCTX-M-53) was grown in 6 liters of brain heart infusion broth containing cefotaxime at 2 μg/ml for 18 h at 37°C. The bacteria collected by centrifugation were suspended with 20 mM MES (morpholineethanesulfonic acid)-NaOH (pH 6.0) and disrupted by ultrasonic treatment (four times for 30 s, each time at 20 W). After centrifugation (10,000 × *g* for 10 min at 4°C), nucleic acids were precipitated by the addition of 0.2 M (7% [vol/vol]) spermine and centrifugation at 48,000 × *g* for 60 min at 4°C. The clarified supernatant was dialyzed overnight against 20 mM MES-NaOH (pH 6.0). The CTX-M purification was carried out as previously described (7) by ion-exchange chromatography with an SP Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden) and gel filtration chromatography with a Superose 12 column (Amersham Pharmacia Biotech). The total protein concentration was estimated by the Bio-Rad protein assay, with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) used as a standard.

The purity of the CTX-M extracts was estimated as previously described (7) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie blue R-250 (Sigma Chemical Co.).

Determination of β-lactamase kinetic constants. The kinetic constants K_m and k_{cat} of the β-lactamases were obtained by a computerized microacidimetric method as previously described (25). The concentrations of the inhibitors (clavulanate and tazobactam) required to inhibit enzyme activity by 50% (IC_{50}s) were determined as described previously with penicillin G (7). The IC_{50}s were monitored with penicillin G (200 mM) as the reporter substrate. The kinetic constants were determined three times. The variation coefficients had a maximum of 10%, except with the CTX-M-9 enzyme for aztreonam and ceftazidime for which it had a maximum of 20%.

Transposition assay. The mobility of a putative transposable element comprising the $bla_{CTX\text{-}M\text{-}53}$ gene was studied by two different methods. (i) A $bla_{CTX\text{-}M\text{-}53}$ transposition assay was done in a standard mating assay as described previously (18). The *E. coli* donor strain HB101 (*recA*, streptomycin resistant) harboring pOX38-Neo^r was transformed with plasmid pWES-1. The resulting strain, HB101(pOX38-Neo^r, pWES-1), was mated with *E. coli* DH1 (Nal^r) either in liquid or on solid medium at different temperatures (25, 30, and 37°C). The transconjugants were selected on Drigalski medium containing Nal (50 μg/ml), kanamycin (40 μg/ml), and cefotaxime (4 μg/ml). (ii) Plasmid pGBG1 was introduced by electroporation into *E. coli* DH10B containing the natural plasmid pWES-1 and also into *S. enterica* isolate 04CEB8273SAL. Plasmid pGBG1 contains an insertion cartridge allowing positive selection for tetracycline resistance. This plasmid is dedicated to the isolation of mobile genetic elements in a wide variety of gram-negative bacteria (35). Tetracycline-resistant mutants were selected on LB plates containing the appropriate tetracycline concentration that prevents spontaneous mutants in pGBG1-free isolates. An analysis of the pGBG1 insertion cartridge was performed by PCR in tetracycline-resistant mutants, as previously described (35).

Nucleotide sequence accession number. The nucleotide sequence of plasmid pWES-1 (11 kb) containing the $bla_{CTX\text{-}M\text{-}53}$ gene has been deposited in the GenBank database under accession no. DQ268764.

RESULTS

Antimicrobial susceptibility. *S. enterica* serotype Westhampton (antigenic formula 3,10:g,s,t:-) and Senftenberg (antigenic formula 1,3,19:g,s,t:-) isolates were resistant to amoxicillin, ticarcillin, piperacillin, cephalothin, cefamandole, ceftazidime, and ceftriaxone and remained in vitro susceptible to piperacillin-tazobactam, cefoxitin, and imipenem by the disk diffusion method. The isolates were susceptible to all other antibiotic classes tested. An ESBL phenotype was detected by using the double disk diffusion test (data not shown) and ESBL detection Etest strips (Table 2). *S. enterica* serotype Westhampton isolate 04CEB8273SAL was highly resistant both to ceftriaxone and ceftazidime (MICs of >256 mg/liter). MICs of other β-lactams are shown in Table 2.

Characterization of the β-lactamase gene(s). CTX-M consensus PCR performed on all the isolates gave the expected PCR product of 540 bp, whereas PCRs for bla_{TEM}, bla_{SHV}, and the $bla_{OXA\text{-}1}$ group were negative. A CTX-M-1 group-specific PCR assay was then carried out, and the isolates gave the expected PCR product of 874 bp. A sequence analysis of the PCR product amplified from the pWES-1 plasmid revealed 99% homology with the corresponding $bla_{CTX\text{-}M\text{-}10}$ gene sequence (GenBank accession number AF255298). To ensure that there were no sequence errors due to primer-directed mismatches at the extremities of the ORF, we determined the entire sequence of the $bla_{CTX\text{-}M}$ gene by genome walking on plasmid pWES-1. This novel $bla_{CTX\text{-}M}$ gene exhibited 99.5% nucleotide identity with the $bla_{CTX\text{-}M\text{-}10}$ gene (AF255298). Its deduced amino acid sequence showed ≥97% identity with the members of the CTX-M-1 phylogenetic group (Fig. 1). This novel CTX-M β-lactamase represented a new member in this group, closely related to CTX-M-10, and thus was named CTX-M-53. The CTX-M-53 protein exhibited different substitutions previously described for CTX-M-1, CTX-M-10, and CTX-M-15 (Fig. 1).

Molecular typing. All four *S. enterica* isolates were tested by XbaI-PFGE. A unique profile, SSFTXB0023, was obtained. Two serotype Westhampton isolates (04CEB8118SAL and 04CEB8273SAL) were tested by MLST and had a sequence type, ST14, frequently found among serotype Senftenberg isolates (http://mlst.ucc.ie/mlst/dbs/Senterica/GetTableInfo_html).

Transfer of β-lactam resistance and plasmid analysis. β-Lactam resistance could not be transferred by conjugation from any *S. enterica* isolate to *E. coli* DH1 (Nal^r), either in liquid or on solid medium. However, β-lactam resistance was transferred to *E. coli* DH10B by electroporation with plasmid DNAs extracted from all *S. enterica* isolates.

A single plasmid of approximately 11 kb was found in all selected *E. coli* transformants (data not shown). The β-lactam-resistant *E. coli* DH10B(pWES-1) exhibited a lower resistance to β-lactams than the parental strain as determined from the

TABLE 2. MICs of β-lactams (Etest) for the *S. enterica* serotype Westhampton isolate 04CEB8273SAL, *E. coli* DH10B harboring natural plasmid pWES-1, *E. coli* DH5α harboring recombinant plasmid pCTX-M-53, *E. coli* DH5α harboring phagemid pBK-CMV, *E. coli* DH10B reference strain, and *E. coli* ATCC 25922 quality control strain

β-Lactam	MIC (μg/ml) for:					
	Serotype Westhampton 04CEB8273SAL	*E. coli* DH10B(pWES-1)	*E. coli* DH5α(pCTX-M-53)	*E. coli* DH5α(pBK-CMV)	*E. coli* DH10B	*E. coli* ATCC 25922
Ampicillin	>256	>256	>256	2	4	4
Amoxicillin-clavulanic acid[a]	8	8	8	4	8	8
Ticarcillin	>256	>256	>256	2	8	8
Ticarcillin-clavulanic acid[b]	128	32	32	2	2	4
Piperacillin	>256	>256	>256	1	2	2
Piperacillin-tazobactam[c]	16	4	2	1	4	2
Cefoxitin	8	4	4	2	4	2
Ceftazidime	>256	64	64	0.125	0.5	0.25
Ceftazidime-clavulanic acid[d]	2	0.5	0.125	0.125	0.5	0.125
Ceftriaxone	>256	>256	>256	<0.06	0.125	0.06
Cefotaxime-clavulanic acid[d]	1	0.125	0.06	<0.06	0.125	0.06
Cefepime	>32	32	>32	<0.06	0.125	0.06
Aztreonam	>256	128	128	<0.06	0.25	0.06
Imipenem	0.5	0.5	0.5	0.25	0.5	0.25

[a] 2:1 amoxicillin-clavulanic acid.
[b] 2 μg/ml clavulanic acid.
[c] 4 μg/ml tazobactam.
[d] 4 μg/ml clavulanic acid.

MIC (Table 2). These plasmids extracted from the different transformants were further characterized by AvaI restriction analysis showing that they were all identical and around 11 kb in size (data not shown).

IEF and kinetic parameters. *S. enterica* isolate 04CEB827 3SAL produced a single β-lactamase with a pI of approximately 8.4 (data not shown). The *E. coli* DH10B transformant (pWES-1) expressed the same β-lactamase (pI, 8.4) (data not shown).

The purified CTX-M proteins appeared on sodium dodecyl sulfate-polyacrylamide gels as a single band (≥97% pure) of 28.6 kDa (data not shown). The substrate profile of CTX-M-53 is shown in Table 3. Kinetic constants exhibited usual values for CTX-M-type ESBLs. K_m values were lower for penicillins (10 to 40 μM) than for cephalosporins (80 to 174 μM). Cephalothin was the best substrate (k_{cat} for cephalothin was 15- to 200-fold higher than those for penicillins). A 40- to 145-fold higher k_{cat} value was observed for cefotaxime than for carboxy propyl oxyimino β-lactams (585 versus 4 to 14 s^{-1}). However, the kinetic parameters were unusual, with a significant k_{cat} value for ceftazidime (14 s^{-1}) and a low K_m value for aztreonam (14 μM). The enzyme was susceptible to tazobactam (IC_{50}, 2.2 nM) and clavulanate (IC_{50}, 10.0 nM).

Genetic environment of the *bla*$_{CTX-M-53}$ gene. To study the genetic environment of the *bla*$_{CTX-M-53}$ gene, we have sequenced the entire pWES-1 (11 kb) on both strands (accession number DQ268764). The plasmid was shown to be 10,908 bp in size and to contain 12 ORFs (Table 4 and Fig. 2). The backbone of the pWES-1 plasmid shares significant nucleotide identity with two different elements (Fig. 2). A 3.3-kb region of pWES-1 shared ≥80% nucleotide identity with a part of the Tn*CP23* transposon found in the chromosomal integrated plasmid pKLC102 of *Pseudomonas aeruginosa* (Table 4 and Fig. 2) (24). A second 4-kb region of pWES-1 shared ≥90% nucleotide identity with two similar plasmids carrying the quinolone resistance gene *qnrS2* from *Aeromonas hydrophila* (GenBank accession number EU925817) and from the bacterial community of a wastewater treatment plant (GenBank accession number DQ460733), respectively (4). These two regions were shown to contain different ORFs and structures implicated in plasmid replication and mobilization (Table 4 and Fig. 2). Between these regions of the plasmid backbone are located the *bla*$_{CTX-M-53}$ gene, ORF477Δ, and IS*26* element on one side and a novel insertion sequence (IS) on the other side (Table 4, Fig. 2). This novel IS of the IS*5* family has been deposited in the IS Finder database (http://www-is.biotoul.fr/is.html) and named IS*Sen2*.

Transposition of the *bla*$_{CTX-M-53}$ gene. The transposition of *bla*$_{CTX-M-53}$ was assayed by using plasmid conjugation and a capture vector system. No transposition event of the *bla*$_{CTX-M-53}$ gene was obtained despite multiple attempts either in conjugation experiments or with the pGBG1 capture system. Thus, it suggests that the plasmid fragment containing the *bla*$_{CTX-M-53}$ gene and flanked by two different ISs (IS*Sen2* upstream and IS*26* downstream) cannot be mobilized by transposition. However, in conjugation experiments, when transconjugants were selected on plates containing only cefotaxime and Nal, numerous transconjugants carrying the pWES-1 plasmid were obtained. This result indicates that the *bla*$_{CTX-M-53}$-carrying plasmid pWES-1 was mobilizable in the presence of a helper plasmid (pOX38-Neo^r) in the donor strain.

DISCUSSION

In our study, we report the characterization of a novel CTX-M-53 ESBL identified in *S. enterica* serotype Westhampton and Senftenberg strains isolated from cockles in France. According to our knowledge, there were only two previous descriptions of CTX-M ESBLs (CTX-M-3) in serotype Senftenberg in Algeria and Japan (1, 29). In the CTX-M-1

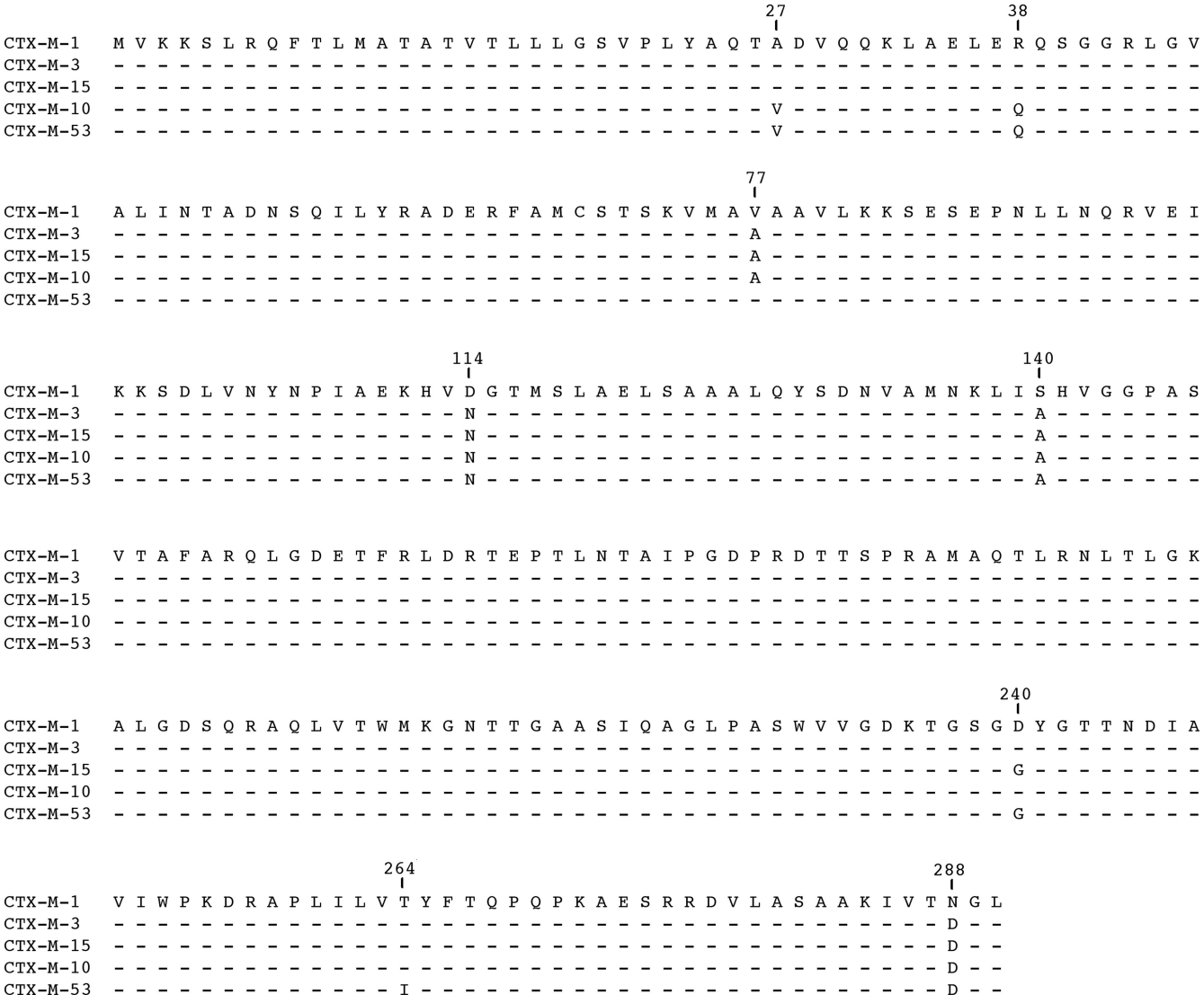

FIG. 1. Alignment of the deduced amino acid sequences encoded by the novel $bla_{CTX\text{-}M\text{-}53}$ gene with those of members of the phylogenetic CTX-M-1 group. Amino acid sequences are from CTX-M-1 (X92506), CTX-M-3 (Y10278), CTX-M-15 (AY044436), CTX-M-10 (AF255298), and CTX-M-53 (DQ268764). The positions of the substitutions are indicated according to the standard numbering scheme for the class A β-lactamases (2). Dashes indicate amino acids identical to those of CTX-M-1.

phylogenetic group, the amino acid sequence of CTX-M-53 is closely related to those of CTX-M-10 (31) and CTX-M-34 (GenBank accession number AY515297), which formed a cluster of CTX-M enzymes characterized by the association of residues Val27 and Q38. The sequence of CTX-M-53 differs from those of CTX-M-10 and CTX-M-34 by substitutions Ala77Val and Asp240Gly and from those of all previously reported CTX-M enzymes by the substitution Tyr264Ile.

TABLE 3. Substrate profiles of the CTX-M-53 β-lactamases

β-Lactamase	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
Penicillin G	135	10	13.5
Amoxicillin	85	40	2.1
Ticarcillin	10	18	0.5
Piperacillin	90	12	7.5
Cephalothin	2,000	122	16.4
Cefuroxime	292	80	3.6
Cefotaxime	585	180	3.2
Cefpirome	710	125	5.7
Ceftazidime	14	174	0.1
Aztreonam	4	14	0.3

The substitution Asp240Gly in CTX-M-53 was previously observed in enzymes CTX-M-15 (33), CTX-M-16 (7), CTX-M-25 (28), CTX-M-27 (8), CTX-M-28 (20), CTX-M-29 (39), CTX-M-33 (17), CTX-M-41 (30), CTX-M-43 (10, 11), CTX-M-55 (23), CTX-M-57 (21), CTX-M-64 (AB284167), CTX-M-69 (EU402393), CTX-M-79 (EF426798), and CTX-M-82 (DQ256091). The kinetic study of enzymes CTX-M-15, CTX-M-16, and CTX-M-27 revealed the implication of this mutation in the improvement of the catalytic efficiency against ceftazidime (k_{cat}/K_m, 0.001, 0.04, and 0.01 $s^{-1} \cdot \mu M^{-1}$, respectively) and of K_m values against aztreonam (K_m, 11, 17, and 17 μM, respectively) (7, 8, 33). The atomic resolution structure of CTX-M-27 showed that the Asp240Gly substitution allowed

TABLE 4. ORFs and other features of $bla_{CTX\text{-}M\text{-}53}$ -carrying plasmid pWES-1

ORF name/ feature	Location (start-stop)[a]	Size in:		G+C content (%)	% Identity/ range[b]	Exhibits significant homology[c] to (accession no.):
		bp	Amino acids			
oriV1	1–539	539	NA[d]	63.4	77/545	*oriV* region; transposon Tn*CP23* of *Pseudomonas aeruginosa* plasmid pKLC102 (AY257539)
IS*Sen2*	557–1402	846	NA	66.2	73/763	Transposase-containing region; *Methylobacterium radiotolerans* plasmid (CP001006)
$tnpA_{ISSen2}$	629–1392	764	254	66.7	47/255	IS*869* putative transposase; *Agrobacterium tumefaciens* Ti plasmid (X53945)
oriV2	1593–1808	216	NA	66.2	95/220	*oriV* region; mobilizable IncQ-related plasmid pGNB2 (DQ460733)
ORF1	2326–1859	468	155	59.6	92/155	Hypothetical protein; plasmid pGNB2 (DQ460733)
nic	2659–2670	12	NA	NA	100/12	Putative *nic* site of *oriT*; plasmid pGNB2 (DQ460733)
mobC	2783–3169	387	128	68.3	74/143	Auxiliary mobilization protein C; mobilizable IncQ-related plasmid pGNB2 (DQ460733)
ORF2	3153–5594	2,442	813	66.8	95/813	Relaxase/mobilization nuclease topoisomerase/primase fusion protein; IncQ-related plasmid pGNB2
$bla_{CTX\text{-}M\text{-}53}$	5892–6767	876	291	54.4	98/291	β-Lactamase CTX-M-57 in *Salmonella enterica* serotype Typhimurium (DQ810789)
ORF477Δ	7227–6813	415	NA	57.1	100/413	ORF477 from *Klebsiella pneumoniae* plasmid pRYCE21 (AY598759)
IS*26*	7228–8047	820	NA	52.3	99/820	IS*26* insertion sequence (X00011)
$tnpA_{IS26}$	7291–7995	705	234	53.7	99/234	Transposase of IS*26* (X00011)
res	8216–8737	522	173	70.1	78/173	Putative resolvase; transposon Tn*CP23* of *Pseudomonas aeruginosa* plasmid pKLC102 (AY257539)
parA	8995–9624	630	209	65.6	97/210	Partitioning protein; transposon Tn*CP23* of *Pseudomonas aeruginosa* plasmid pKLC102 (AY257539)
parB	9645–9854	210	69	57.6	78/73	Putative plasmid stabilization protein; transposon Tn*CP23* of *Pseudomonas aeruginosa* plasmid pKLC102 (AY257539)
repA	9907–10908	1,002	333	64.3	91/337	Replication protein; transposon Tn*CP23* of *Pseudomonas aeruginosa* plasmid pKLC102 (AY257539)

[a] Nucleotide position in the sequence deposited under accession no. DQ268764.
[b] Percentage of identity as returned by BLAST search and range (number of amino acids or nucleotides and gaps) over which the identity value was calculated. Percentage of identity as returned by BLASTN search and range for *oriV1*, *oriV2*, *oriT*, and IS elements.
[c] Significant homology is defined as >20% identity over at least 60% of the length of the protein.
[d] NA, not applicable.

broad and coordinated vibrations of the β3 strand (14). Molecular modeling experiments also suggest coordinated motions of the β3 strand with the reactive Ser70 and residues 167 to 170 of the Ω loop which are critical for cephalosporin accommodation (14, 15). This behavior of the binding site and the absence of a negative-charged residue at position 240 probably allowed a deep insertion of ceftazidime in the catalytic pocket, as observed for a ceftazidime-like compound in the crystal structure of CTX-M-16 (14, 15). These different modifications explained the increased activity against ceftazidime observed in the Gly240-harboring CTX-M enzymes.

The residue Tyr264 is conserved in CTX-M enzymes. In the crystallographic structure of CTX-M-9, its side chain is located in the core of the protein and in contact with the CTX-M conserved residues Thr71, Val262, and Met186 (13–15). The atomic resolution of this structure revealed double conformations of the three residues Met186, Val262, and Tyr264, suggesting the high mobility of their side chains (14). In CTX-M-

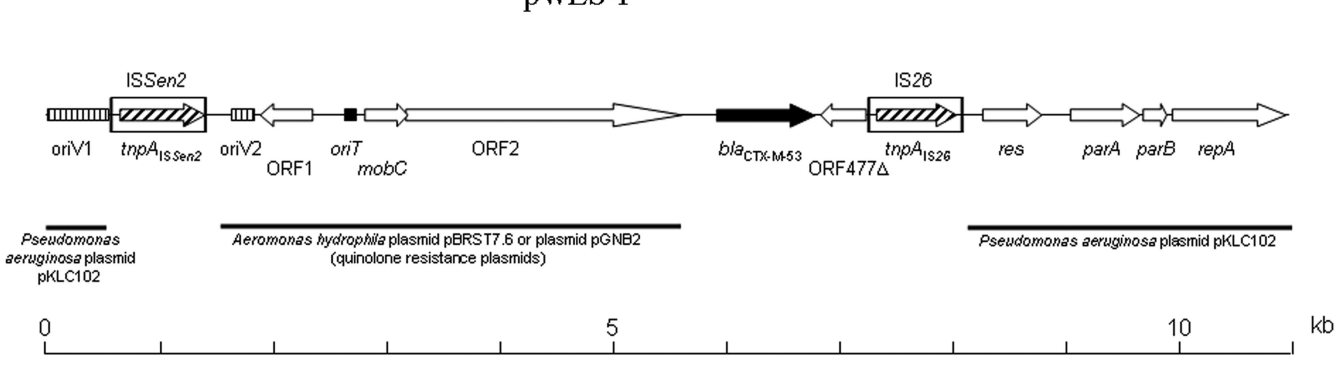

FIG. 2. Linear representation of pWES-1 (10.9 kb) harboring the $bla_{CTX\text{-}M\text{-}53}$ gene. IS elements are indicated by hatched arrows within boxes. The $bla_{CTX\text{-}M\text{-}53}$ gene is indicated by a black arrow. Vertically striped and black boxes indicate *oriV* and *oriT* regions, respectively. Regions exhibiting significant homology to extant sequences on plasmids and a distance scale are given below the map.

53, the replacement of this residue by Ile264, which is a smaller residue than Tyr264, may increase the mobility of the enzyme in this zone. This zone is located at the vicinity of the ^{70}SXX^{73}K conserved sequence harboring the active serine. Mobility in this zone may therefore influence the catalytic activity of CTX-M-53.

The sequence analysis of plasmid pWES-1 revealed that the plasmid backbone is composed of two different modules for replication initiation and mobilization, respectively. The replication module consists of the *parA*, *parB*, and *repA* genes, and a large part of the *oriV* region found in an IS*6100* composite transposon integrated in the genomic island pKLC102 from *Pseudomonas aeruginosa* (24). The mobilization module harbors a *mobC* gene, an ORF2 coding for a putative relaxase protein, a putative *oriT* region, and an IncQ-like *oriV* region. This module has been previously described for the IncQ-related plasmid pGNB2 carrying the quinolone resistance gene *qnrS2* (4). These two modules are separated by the novel IS*Sen2* element. The 846-bp length IS*Sen2* encodes two partially overlapping ORFs with a potential site for −1 frameshifting between both ORFs (5′-AAAAGGGGGGA-3′). This novel IS showed several similarities with IS elements of the IS*427* subgroup in the IS*5* family (12, 27): (i) 16-bp inverted repeats, (ii) TA target site duplication, and (iii) the putative transposase of IS*Sen2*, which shared ≥40% identity with several members of this subgroup. A preferred target sequence, YTAR (often CTAG), is observed for two subgroups, IS*5* and IS*427*. Thus, the TA target duplication found outside the inverted repeats suggested that this IS*Sen2* element was inserted alone in the plasmid backbone.

Immediately upstream of the $bla_{CTX\text{-}M\text{-}53}$ gene, any genetic structure could explain its acquisition. Interestingly, the region immediately upstream of $bla_{CTX\text{-}M\text{-}53}$ showed 99% identity with the 74-bp and 118-bp regions located just upstream of $bla_{CTX\text{-}M\text{-}3}$ and $bla_{CTX\text{-}M\text{-}10}$, respectively (32). The acquisition of the $bla_{CTX\text{-}M\text{-}53}$ gene by the pWES-1 plasmid could be the result of a transposition event related to the downstream IS*26* element. A possible explanation is that an IS*26* element was originally present in the pWES-1 plasmid and a recombination event may have occurred between the IS*26* elements resulting in the integration of the $bla_{CTX\text{-}M\text{-}53}$ gene and ORF477Δ. Another possibility is that an IS*26* composite transposon carrying the $bla_{CTX\text{-}M\text{-}53}$ gene and ORF477Δ formed a cointegrate with the pWES-1 plasmid. Then, after a resolution step which is required to separate the donor and target replicons, the upstream copy of IS*26* could have been lost (12, 27).

Considering the origin of CTX-M-53-producing serotype Westhampton and Senftenberg isolates (i.e., living cockles from France), it is relevant to note that this $bla_{CTX\text{-}M\text{-}53}$-carrying plasmid harbored similarities with *qnrS2*-carrying plasmids from aquatic sources (4). Thus, the natural aquatic environment may contribute to genetic exchanges between different bacterial pathogens.

CTX-M-53-producing *S. enterica* isolates were indistinguishable by PFGE, whereas they were of different serotypes. Serotype Westhampton differs from serotype Senftenberg by the type of O antigens, E1 group (O:3,10) or E4 group (O:1,3,19), respectively. It has been demonstrated that the group E1 and E4 strains have the same chromosomal *rfb* gene cluster (encoding the enzymes for O-antigen biosynthesis), and the difference between E1 and E4 was proposed to be due to the presence of a gene(s) on a converting phage in E4, although the phage has not been observed (40). The presence of MLST type ST14, which is common in Senftenberg isolates, in the serotype Westhampton isolates under study also speaks in favor of the close genetic relationship between these two serotypes.

In 2004, no human infections due to *S. enterica* serotypes Westhampton or Senftenberg isolates resistant to ESC were reported by the French National Reference Center for *Salmonella*. It might be due to the fact that cockles are generally cooked before consumption.

In conclusion, this study reported for the first time the identification of the novel $bla_{CTX\text{-}M\text{-}53}$ gene in *Salmonella*, an important food-borne pathogen. It was located on an 11-kb mobilizable plasmid present in serotype Westhampton and Senftenberg isolates from living cockles in France. The CTX-M-53 enzyme harbors substitutions probably implicated in a greater efficiency against ceftazidime and aztreonam than other CTX-M enzymes. The spread of plasmid-mediated CTX-M-producing strains of *Salmonella* is of concern, and an enhanced surveillance of ESBL-producing strains should be performed in animals as well as in humans.

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