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New Extended-Spectrum TEM-Type β-Lactamase from *Salmonella enterica* subsp. *enterica* Isolated in a Nosocomial Outbreak

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A new extended-spectrum β-lactamase was detected in a lactose-positive *Salmonella enterica* subsp. *enterica* strain that caused a nosocomial outbreak involving eight patients in a pediatric cardiology unit. This strain showed high levels of resistance to ceftazidime and aztreonam and relatively low levels of resistance to cefotaxime and ceftriaxone. Resistance was associated with a conjugative plasmid of 59 kb, which encoded a new β-lactamase with an isoelectric point of 5.9 that strongly hydrolyzed ceftazidime and to a much lesser extent hydrolyzed cefotaxime. The enzyme activity was inhibited by clavulanate. The corresponding *bla* gene was cloned and sequenced. The deduced amino acid sequence showed three significant amino acid replacements with respect to the TEM-1 sequence: Arg-164→His, Glu-240→Lys, and Thr-265→Met. This combination is unique among extended-spectrum β-lactamases and served to characterize the new enzyme, TEM-27.

The spread of the new extended-spectrum β-lactamases among members of the family *Enterobacteriaceae* represents one of the major challenges for the future of therapy with the newest cephalosporin antibiotics. These enzymes, generally plasmid mediated, are involved in outbreaks of resistant nosocomial pathogens. Although *Klebsiella* is the genus that most frequently harbors extended-spectrum β-lactamases, these enzymes have also been found in the genera *Escherichia*, *Proteus*, *Morganella*, *Enterobacter*, *Citrobacter*, and *Serratia* (3, 11, 14, 17, 19, 21, 24, 27). Detection of extended-spectrum β-lactamases among members of the genus *Salmonella* is rare. The first strains were detected in France in 1984 and 1987 (*Salmonella typhimurium* with SHV-2); this was followed by the detection of other such strains in Tunisia (*Salmonella wien* with SHV-2) (1988), Martinique (*Salmonella panama* with CTX-1 [now TEM-3]) (1989), France (*Salmonella kedougou* with TEM-3) (1989), Algeria (*Salmonella mbandaka* with CTX-2) (1990), and Argentina (*S. typhimurium* with CTX-M2) (1991) (1, 2, 4, 7, 15, 25). In this report we describe a novel TEM-type β-lactamase, named TEM-27, with high-level ceftazidime-hydrolyzing activity found in a strain of *Salmonella enterica* subsp. *enterica* during a nosocomial outbreak. The epidemic involved eight children hospitalized in the Pediatric Cardiology Unit of the Ramón y Cajal Hospital in Madrid, Spain.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *S. enterica* subsp. *enterica* RYC36536, isolated from a blood culture, was randomly selected from the outbreak isolates. Biochemical and serological confirmation of the identity of this prototype strain was done at the Centre National de Référence des Salmonella et Shigella, Institut Pasteur, Paris, France. *Escherichia coli* K-12 strains BM21 (λ^+ *gyrA*), RYC1000 (F^- *araD139* Δ*lacU169* Δ*rib-7 thiA gyrA recA56*), and TG1 [Δ(*lac-pro*) *supE thi hsdDS*/F′ *traD36 proA*$^+$*B*$^+$ *lacI lacZ*M15] were used as hosts for plasmids. Plasmids pBGS18$^-$ (33) and pACYC184 (9) were used as cloning vectors.

Susceptibility testing. Disk diffusion tests and MICs determinations by agar dilution procedures were performed according to standard guidelines (22, 23). The antibiotics were provided as follows: amoxicillin, ticarcillin, temocillin, ceftizoxime, and clavulanic acid, SmithKline Beecham Pharmaceuticals, Brockham Park, United Kingdom; piperacillin and tazobactam, Cyanamid-Lederle Laboratories, Pearl River, N.Y.; cephaloridine, cefuroxime, and ceftazidime, Glaxo Group Research Ltd., Greenford, United Kingdom; cefazolin, moxalactam, and tobramycin, Eli Lilly & Co., Indianapolis, Ind.; cefoxitin and imipenem, Merck Sharp & Dohme, West Point, Pa.; cefotetan, Stuart Pharmaceuticals, Wilmington, Del.; ceftriaxone and carumonam, Hoffmann-La Roche Inc., Nutley, N.J.; cefotaxime and cefpirome, Hoechst Roussel Pharmaceuticals, Inc., Somerville, N.J.; aztreonam, cefepime, kanamycin, and amikacin, Bristol-Myers Squibb Co., Princeton, N.J.; netilmicin and gentamicin, Schering-Plough Research, Bloomfield, N.J.; meropenem, Zeneca Pharmaceuticals, Macclesfield, United Kingdom; and chloramphenicol, tetracycline, and trimethoprim, Sigma Chemical Co. St. Louis, Mo.

β-Lactamase assays. Cell-free lysates were obtained by ultrasonication of exponentially growing cultures at 37°C in Luria-Bertani broth containing 10 μg of ceftazidime per ml. Analytical isoelectric focusing was done by applying the crude sonic extracts to Phast gels (pH gradients of 3 to 9 and 4 to 6.5) in a Phast system (Pharmacia AB, Uppsala, Sweden). β-Lactamases with known pIs were focused in parallel with the extracts, using nitrocefin (Oxoid Ltd., Basingstoke, Hampshire, England) for detection. The β-lactamase was partially purified, by Sephadex G-75 chromatography in 0.1 M phosphate buffer (pH 7.2), from a crude extract of a 1-liter culture of *E. coli* RYC1000 containing plasmid pJMM3. Fractions containing β-lactamase activity were pooled and concentrated with a Minicon concentrator (Amicon B15; W. R. Grace and Co., Danvers, Mass.). This preparation was stored for a maximum of 48 h at −20°C and used for the determination of kinetic constants. Hydrolysis of β-lactam antibiotics was monitored at 25°C in 0.1 M phosphate buffer (pH 7.2) with a UVIKON-940 spectrophotometer. One unit of enzymatic activity was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate in 1 min at 25°C in 0.1 M sodium potassium buffer (pH 7.2). For specific enzyme activities, protein concentrations were determined by the method of Lowry et al. (18). β-Lactamase activities were determined by measuring the decrease in absorbance for the following antibiotics at the indicated wavelengths: cephaloridine, 275 nm; ceftazidime, 260 nm; cefotaxime, 265 nm; and aztreonam, 318 nm. Kinetic parameters were obtained in at least duplicate experiments by making linear plots of the initial steady-state velocities at different substrate concentrations. Relative V_{max} values and relative V_{max}/K_m values were calculated for comparison of the enzyme activities (6).

Maxicell experiments. Maxicell experiments were performed with strain RYC1000 (*recA*) as described previously (29).

Transfer of resistance and plasmid analysis. *S. enterica* subsp. *enterica* RYC36536 was conjugated with *E. coli* K-12 BM21. Transconjugants were selected on Luria-Bertani agar plates containing 40 μg of nalidixic acid (Winthrop Laboratories, Div. Sterling Drug Inc., New York, N.Y.) per ml and 15 μg of ceftazidime per ml.

Recombinant DNA techniques. Isolation of plasmid DNA, transformation, restriction endonuclease digestion, ligation, agarose gel electrophoresis, and other standard recombinant DNA techniques were performed as described by Sambrook et al. (28). Nucleotide sequencing was carried out for both DNA

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TABLE 1. β-Lactam MICs in *S. enterica* subsp. *enterica* RYC36536, *E. coli* K-12 BM21, and a BM21 derivative producing TEM-27

Antibiotic	MIC (μg/ml) in:		
	S. enterica RYC36536	*E. coli* BM21	*E. coli* BM21 TEM-27
Amoxicillin	>1,024	1	>1,024
Ticarcillin	>1,024	2	>1,024
Piperacillin	>1,024	2	>1,024
Amoxicillin-clavulanic acid	8/4	1/0.5	8/4
Ticarcillin-clavulanic acid	32/2	2/2	32/2
Piperacillin-tazobactam	16/2	2/0.2	8/1
Temocillin	16	4	16
Cefazolin	32	2	32
Cefuroxime	4	4	8
Cefoxitin	2	2	2
Cefotetan	0.2	0.03	0.2
Cefotaxime	2	0.06	0.5
Ceftizoxime	1	0.06	0.5
Ceftriaxone	2	0.06	0.5
Ceftazidime	512	0.2	64
Cefpirome	8	0.01	1
Cefepime	4	0.01	0.5
Aztreonam	64	0.06	32
Carumonam	4	0.01	2
Moxalactam	0.2	0.03	0.2
Imipenem	0.2	0.1	0.1
Meropenem	0.03	0.03	0.03

strands by the dideoxynucleotide chain termination method (30) with T7 DNA polymerase (P-L Biochemicals, Milwaukee, Wis.) and bla_{TEM}-specific primers. The sense primers had the following nucleotide positions in the TEM-1 sequence (34): −210 to −195, 100 to 117, 339 to 356, 474 to 491, and 699 to 716. For the antisense primers, the positions were 986 to 968, 716 to 699, 491 to 474, 356 to 339, 117 to 100, and 18 to 1.

RESULTS

Epidemiology. In February 1989, a lactose-positive *S. enterica* subsp. *enterica* strain, fully susceptible to β-lactam antibiotics and aminoglycosides, was isolated from a blood culture from a newborn patient recently admitted to the Pediatric Cardiology Unit of the Hospital Ramón y Cajal in Madrid, Spain. In June 1989, a ceftazidime-resistant, lactose-positive *S. enterica* subsp. *enterica* strain was isolated from blood cultures and bronchial secretions from a newborn hospitalized in the same unit. In the subsequent weeks, 19 ceftazidime-resistant, lactose-positive *S. enterica* subsp. *enterica* strains were isolated from seven other patients hospitalized in the same unit. The resistant organisms were cultured from blood (1 isolate), catheters (2 isolates), surgical wound secretions (2 isolates), stool samples (10 isolates), and respiratory tract specimens (9 isolates). All eight patients were previously treated with antibiotics, most frequently with aminopenicillin or cefazolin plus an aminoglycoside (tobramycin or gentamicin).

The epidemic was controlled at the end of July after restriction of the use of ceftazidime and cefotaxime (imipenem was used as an alternative), stringent isolation of patients and carriers, emphasis on handwashing procedures, and intensive disinfection of the unit.

Resistance phenotype of *S. enterica* subsp. *enterica* RYC36536. Table 1 shows the MICs of β-lactams obtained for the *S. enterica* subsp. *enterica* RYC36536 strain. Ceftazidime and aztreonam had high MICs (512 and 64 μg/ml, respectively), while the strain had relatively low levels of resistance to cefotaxime (MIC, 2 μg/ml), ceftriaxone (2 μg/ml), and ceftizoxime (1 μg/ml). This strain was also resistant to aminoglycosides, as was previously shown (12). All isolates remained susceptible to tetracycline, chloramphenicol, trimethoprim, nalidixic acid, and fluoroquinolones.

Plasmid characterization. *S. enterica* RYC36536 transferred to *E. coli* BM21 by conjugation a single plasmid (pJMM1), as determined by gel electrophoresis. Transconjugants acquired the complete resistance pattern of the donor (Table 1), and their lysates showed a single β-lactamase band of pI 5.9 in isoelectrofocusing gel electrophoresis. The plasmid size was estimated to be 59 kb by agarose gel electrophoresis, as determined by the sum of the sizes of fragments obtained after endonuclease digestion. Identical plasmid DNA restriction profiles were obtained for plasmids of other *S. enterica* strains isolated in the same outbreak.

Cloning of the bla_{TEM-27} gene of pJMM1. Plasmid pJMM1 DNA was digested with *Sac*I, and the resulting fragments were ligated with pBGS18$^-$ previously digested with *Sac*I. The ligation mixture was used to transform *E. coli* RYC1000 with selection for ceftazidime and kanamycin resistance. A plasmid, designated pJMM2, that contained a 5.5-kb *Sac*I fragment from pJMM1 including its *bla* gene was isolated from transformants. pJMM2 was digested with *Ssp*I and *Sal*I, and the resulting fragments were ligated to *Eco*RV-*Sal*I-digested pACYC184. The plasmid obtained, named pJMM3, harbored an insert of 2.1 kb which conferred resistance to ceftazidime. Analytical isoelectric focusing indicated that pJMM3 specified a β-lactamase with a pI of 5.9.

DNA sequencing. The complete sequence of the bla_{TEM} gene encoding the β-lactamase responsible for ceftazidime resistance, including the promoter sequence, was obtained with pJMM2 and pJMM3 by using the bla_{TEM-1}-specific primers described in Materials and Methods. The comparison of the nucleotide and deduced amino acid sequences with those of TEM-1 β-lactamases revealed three substitutions: Arg-164→His (CGT→CAT), Glu-240→Lys (GAG→AAG), and Thr-265→Met (ACG→ATG). This combination of amino acid changes is unique among TEM-type β-lactamases, and it is therefore proposed to denote this new enzyme TEM-27. The sequence of the gene encoding TEM-27 showed other nucleotide changes that did not result in alterations of the amino acid sequence compared with that of TEM-1A (34); these changes were in codon 78 (GGC→GGT), codon 160 (ACT→ACC), and codon 242 (GGG→GGA).

Physical and kinetic properties of the β-lactamase. Analytical isoelectric focusing of crude extracts from *S. enterica* subsp. *enterica* RYC36536 revealed a single β-lactamase band at pI 5.9. An identical band was found in crude extracts from the other 23 isolates of *S. enterica* subsp. *enterica*. The apparent molecular weight of the TEM-27 β-lactamase was almost identical to that of TEM-1 (M_r, 29,000) according to data obtained from maxicell experiments with RYC1000 harboring pJMM3 (data not shown). Values of kinetic constants for the β-lactamase studied are shown in Table 2. The predominant ceftazidime-hydrolyzing activity was related to a higher hydrolysis rate of the enzyme with ceftazidime than with cefotaxime, which resulted in a proportionally higher hydrolytic efficiency.

DISCUSSION

The results obtained in this work document the emergence of a new TEM-type β-lactamase, TEM-27, with high-level ceftazidime-hydrolyzing activity, in *S. enterica* subsp. *enterica*. The spread of this type of extended-spectrum β-lactamase

TABLE 2. Kinetic constants of TEM-27 β-lactamase

Antibiotic	K_m (μM)	Relative V_{max} [a]	Relative V_{max}/K_m [a]
Cephaloridine	52	100	100
Cefotaxime	30	5.1	8.9
Ceftazidime	39	65	86.5
Aztreonam	27	6	11.5

[a] Obtained by normalizing the value for each antibiotic to that for cephaloridine (taken as 100).

among *Salmonella* spp. is of particularly concern. In developing countries, the epidemic potential of the genus *Salmonella* may contribute to the dispersion of these resistance genes even in the absence of antibiotic selective pressure.

The combination of amino acid replacements found in TEM-27 is unique, but the changes have been previously documented in other extended-spectrum enzymes. The change Arg-164→His was found in TEM-6, TEM-11, and TEM-16 (8, 13, 19). The phenotypic consequences of this replacement have never been studied in the absence of other changes; it is conceivable that this mutation would have an effect similar to that of Arg-164→Ser. The replacement Arg-164→Ser (TEM-12) produces an increase in the MIC of ceftazidime but only a slight increase in the MIC of cefotaxime (5, 19, 32). The change Glu-240→Lys was found in TEM-5, TEM-10 (or TEM-23), and TEM-24 (8, 26, 31). When this replacement was studied in the absence of other changes (5, 32), the phenotypic results was a slight increase in ceftazidime (but not cefotaxime) resistance. A combination of the changes Arg-164→His and Glu-240→Lys could produce a more significant increase in the ceftazidime MIC. In fact, it was previously shown that the substitution by lysine in position 240 enhances the effect of Ser-164 substitution on the catalytic efficiency on ceftazidime in a modified TEM-1 engineered derivative (32), and this combination explains the ceftazidime-hydrolyzing activity of TEM-10 (26). The change Thr-265→Met was found in TEM-4, TEM-9, TEM-13, and TEM-14 (16). A specific artificial construction containing this replacement does not modify the susceptibility to cephalosporins (5). Considering all available data, at present it is difficult to determine the possible evolutionary origin of the new TEM-27 enzyme. In general, the changes detected suggest that the evolution towards TEM-27 started from the gene encoding TEM-1B (10, 20).

In summary, a new TEM-type extended-spectrum β-lactamase, TEM-27, was found in a strain of *S. enterica* subsp. *enterica* isolated in a pediatric cardiology unit. The spread of extended-spectrum β-lactamases to bacterial genera with significant abilities to cause epidemics is particularly worrisome.

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