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Ceftazidime-Resistant *Enterobacteriaceae* Isolates from Three Polish Hospitals: Identification of Three Novel TEM- and SHV-5-Type Extended-Spectrum β-Lactamases

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Twelve ceftazidime-resistant isolates of the family *Enterobacteriaceae* (11 *Klebsiella pneumoniae* isolates and 1 *Escherichia coli* isolate) were collected in 1995 from three Polish hospitals located in different cities. All were identified as producers of extended-spectrum β-lactamases (ESBLs). Detailed analysis of their β-lactamase contents revealed that six of them expressed SHV-5-like ESBLs. The remaining six were found to produce three different TEM enzymes, each characterized by a pI value of 6.0 and specified by new combinations of amino acid substitutions. The amino acid substitutions compared to the TEM-1 β-lactamase sequence were Gly238Ser, Glu240Lys, and Thr265Met for TEM-47; Leu21Phe, Gly238Ser, Glu240Lys, and Thr265Met for TEM-48; and Leu21Phe, Gly238Ser, Glu240Lys, Thr265Met, and Ser268Gly for TEM-49. The new TEM β-lactamases, TEM-47, TEM-48, and TEM-49, belong to a subfamily of TEM-2-related enzymes. Genes coding for TEM-47 and TEM-49 could have originated from the TEM-48-encoding sequence by various single genetic events. The new TEM derivatives probably document the already advanced microevolution of ESBLs ongoing in Polish hospitals, in a majority of which no monitoring of ESBL producers was performed before 1996.

Extended-spectrum β-lactamases (ESBLs) are enzymes manifesting considerable hydrolyzing activity on a wide variety of β-lactam antibiotics including oxyiminocephalosporins and aztreonam. The genes coding for ESBLs are usually carried by plasmids, which strongly facilitates their spread among strains of many species of gram-negative bacteria. Nevertheless, *Klebsiella pneumoniae* and *Escherichia coli* remain the most frequently reported producers of ESBLs (12, 22).

Since the first ESBL-expressing isolate was discovered in Germany 15 years ago (24, 25) many kinds of ESBLs have been characterized and described. They exhibit high degrees of diversity in their structures and activities, and several families reflecting their evolutionary and/or functional similarities can be distinguished (13, 22).

Among the most prevalent types of ESBLs are members of the TEM and SHV families which are related to the widespread, plasmid-borne, broad-spectrum β-lactamases TEM-1 or TEM-2 and SHV-1, respectively. The TEM and SHV ESBLs exhibit a considerable variety, mostly with respect to their ranges of substrate preferences and to their levels of hydrolytic activity (13, 22). The functional diversity of TEM and SHV β-lactamases is determined by differences in their structures. To date about 15 amino acid positions in ESBL protein sequences have been reported to be heterogeneous (26). Mutations in some of these are critical for expanding the substrate profiles of the enzymes or raising their hydrolyzing activities. Such substitutions are efficiently selected under the pressure of β-lactam use. Other described mutations have no influence on β-lactamase activity, and as neutral substitutions they do not undergo any selective constraints. Together with silent mutations observed in genes coding for β-lactamases, they provide information about the degree of relatedness and the evolutionary lines within ESBL families (26). Isolation and sequencing of natural genes encoding ESBLs provide important data from an epidemiological point of view and contribute to our understanding of the structures and functions of β-lactamases.

Little is known about the prevalence and types of ESBLs expressed by bacterial strains present in the clinical environments of Central and Eastern Europe. In the study reported here we investigated the types of ESBLs produced by a group of 12 isolates of the family *Enterobacteriaceae* (11 *K. pneumoniae* isolates and 1 *E. coli* isolate) collected in three Polish hospitals at the beginning of 1995. Six of these were identified as being producers of SHV-5-like β-lactamases, and the remaining six were found to express three new sequence variants of TEM derivatives; these three variants are likely to form a subfamily of highly related enzymes within the group of TEM β-lactamases.

MATERIALS AND METHODS

Clinical isolates. Eleven *K. pneumoniae* isolates and 1 *E. coli* isolate resistant to ceftazidime were collected from three hospitals located in different cities (the Polish Mother Memorial Hospital in Łódź, Voivodship Hospital No. 2 in Rzeszów, and the Bródno Voivodship Hospital in Warsaw) between January and April 1995. Clinical data concerning the isolates are presented in Table 1. Two *K. pneumoniae* isolates from Rzeszów isolates, L-380 and L-577, were collected from the same patient at an interval of 22 days, during which time the patient was moved from the pediatric ward to the surgical ward. Almost all patients were receiving antibiotics before the isolation of bacterial strains; five of the patients were receiving ceftazidime and one was receiving cefuroxime. Identification of the species of the isolated strains and preliminary determinations of their susceptibility patterns were performed by the clinical microbiological laboratories at the hospitals. The identification of species was later confirmed by the ID32E ATB test (BioMerieux), and all the strains were identified as ESBL producers by the double-disc test (23).

Typing by RAPD analysis. Genomic DNA for typing was extracted from bacterial cells with the Genomic DNA Prep Plus kit (A & A Biotechnology, Gdańsk, Poland). Randomly amplified polymorphic DNA (RAPD) analyses were performed with RAPD-7 (39) and ERIC-1 (42) oligonucleotides as primers. A single reaction mixture contained about 10 ng of genomic DNA, 50 pmol of a

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primer, 100 μM (each) deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 10 μg of bovine serum albumin, 2 U of *Taq* polymerase (MBI Fermentas), and buffer supplied by the manufacturer of the enzyme. Reactions were run with a GeneAmp PCR System 2400 (Perkin-Elmer) under the following conditions: 5 min at 94°C, followed by 5 cycles of 15 s at 94°C, 30 s at 35°C, and 1.5 min at 72°C; 30 cycles of 15 s at 94°C, 15 s at 55°C, and 30 s at 72°C; and finally, 7 min at 72°C. The PCR products were electrophoresed in 2% agarose gels (NuSieve 3:1; FMC Bioproducts).

Susceptibility testing. The MICs of the various antibiotics were determined by the agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards (32). The following antibiotics were used: ampicillin and cefotaxime (Polfa, Tarchomin, Poland), aztreonam (Bristol-Myers Squibb, New Brunswick, N.J.), cefoxitin (Sigma Chemical Company, St. Louis, Mo.), ceftazidime (Glaxo Wellcome, Stevenage, United Kingdom), lithium clavulanate (SmithKline Beecham Pharmaceuticals, Betchworth, United Kingdom), imipenem (Merck Sharp & Dohme Research, Rahway, N.J.), piperacillin (Lederle Inc., Carolina, Puerto Rico), and tazobactam (Lederle Laboratories, Pearl River, N.Y.). For all β-lactam–inhibitor combinations the constant concentrations of clavulanate and tazobactam were 2 and 4 μg/ml, respectively. *E. coli* ATCC 25922 was used as the reference strain for antimicrobial susceptibility testing.

Resistance transfer. A ceftazidime resistance transfer experiment was carried out with all isolates. *E. coli* A15 R^- resistant to nalidixic acid was used as the recipient strain. Equal volumes (1 ml) of cultures of the donor and the recipient strains (10^9 CFU of each strain per ml) grown in tryptic soy broth (Oxoid) were mixed, and the mixtures were incubated for 18 h at 35°C. Transconjugants were selected on MacConkey agar (Oxoid) supplemented with nalidixic acid (64 mg/liter; Sigma Chemical Company) to inhibit the growth of the donor strain and ceftazidime (2 mg/liter) to inhibit the growth of the recipient strain.

IEF of β-lactamases. Crude preparations of β-lactamases (4) were subjected to isoelectric focusing (IEF) by the procedure of Matthew et al. (29), with modifications (4), by using an LKB Multiphor apparatus (Pharmacia LKB). Following IEF, β-lactamase bands were visualized by staining with nitrocefin (Oxoid). Gels were run over a pH range of 3 to 10.

Bioassays for the detection of ceftazidime-hydrolyzing activity. After IEF ceftazidime-hydrolyzing activity was assigned to visualize β-lactamase bands by the bioassay approach described previously (6). The ceftazidime concentration used in the experiment was 2 μg/ml.

PCR detection of bla_{TEM} and bla_{SHV} genes. Plasmid DNA extracted from transconjugant cells was used as templates in specific PCRs for the detection of bla_{TEM} and bla_{SHV} genes. Plasmid DNA was purified from bacterial cells by the alkaline lysis method (8) with the QIAGEN Plasmid Midi Kit (QIAGEN, Hilden, Germany) as described previously (6). TEM-A and TEM-B primers (28) were used for the amplification of the bla_{TEM} genes; SHV-A (5′-ACTGAATG AGGCGCTTCC-3′) and SHV-B (5′-ATCCCGCAGATAAATCACC-3′) primers were used for the amplification of genes coding for SHV β-lactamases (7). A single reaction mixture contained about 1 μg of plasmid DNA, 50 pmol of each primer, 100 μM (each) deoxynucleoside triphosphates, 1 U of *Taq* polymerase (Boehringer Mannheim) and buffer with 2.5 mM $MgCl_2$ supplied by the manufacturer of the enzyme. A Perkin-Elmer 9600 apparatus was used, and the reactions were run under the following conditions: 3 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 42°C, and 1 min at 72°C and, finally, 3 min at 72°C for the bla_{TEM} amplification and 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C and, finally, 3 min at 72°C for the bla_{SHV} amplification. The resulting PCR products were run in 1% agarose gels (SeaKem; FMC Bioproducts).

Sequencing of TEM and SHV PCR products. Specific PCR products were purified with a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and were subjected to direct sequencing reactions by the dideoxy chain termination method of Sanger et al. (35) with an automatic sequencer (373A; Applied Biosystems, Weiterstadt, Germany). The TEM-A, TEM-B, TEM-C, TEM-D, and TEM-E primers (28) were used to sequence the amplified bla_{TEM} genes. The SHV-A and SHV-B primers were used to sequence the amplified parts of the bla_{SHV} genes.

Nucleotide sequence accession numbers. The nucleotide sequence data reported here will appear in the EMBL database under the following accession numbers: Y10279 for the bla_{TEM-47} gene, Y10280 for the bla_{TEM-48} gene, and Y10281 for the bla_{TEM-49} gene.

RESULTS

Typing. The *K. pneumoniae* isolates were typed by the RAPD approach, and the results are presented in Table 1. Both primers were found to be equally discriminative. Four isolates from the hospital in Rzeszów (isolates L-372, L-380, L-491, and L-577) had identical RAPD patterns (pattern B). All the other strains, including the subgroup of five *K. pneumoniae* isolates from the hospital in Łódź, had different RAPD patterns.

TABLE 1. Clinical data, RAPD patterns, IEF of β-lactamases, and identified β-lactamases of isolates of the family *Enterobacteriaceae* and transconjugants

Strain	Species	Date of isolation (day.mo.yr)	Type of specimen	Hospital, ward	Type of infection	RAPD pattern	Isoelectric points of β-lactamases	β-Lactamase identified by sequence analysis
L-267	*K. pneumoniae*	18.1.1995	Throat swab	Łódź, pediatric	Pneumonia, pharyngitis	A	6.0,[a] 7.6	TEM-47
L-372	*K. pneumoniae*	26.1.1995	Urine	Rzeszów, pediatric	UTI[b]	B	<5.4, 6.0, 7.6	TEM-48
L-380	*K. pneumoniae*	1.2.1995	Urine	Rzeszów, pediatric	UTI	B	<5.4, 6.0, 7.6	TEM-48
L-491	*K. pneumoniae*	15.2.1995	Urine	Rzeszów, pediatric	UTI	B	<5.4, 6.0, 7.6	TEM-48
L-577	*K. pneumoniae*	23.2.1995	Urine	Rzeszów, surgical	UTI	B	<5.4, 6.0, 7.6	TEM-48
L-439	*K. pneumoniae*	4.2.1995	Urine	Rzeszów, pediatric	UTI	C	<5.4, 7.6, 8.2	SHV-5-like
L-414	*K. pneumoniae*	24.1.1995	Tracheostomy tube	Łódź, ICU[c]	Suspected pneumonia	D	<5.4, 7.6, 8.2	SHV-5-like
L-251	*K. pneumoniae*	9.1.1995	Bronchial exudate	Łódź, pediatric	Pneumonia	E	5.4, 7.6, 8.2	SHV-5-like
L-95	*K. pneumoniae*	4.1.1995	Wound swab	Warsaw, neurosurgical	Wound infection	F	7.6, 8.2	
L-352	*K. pneumoniae*	22.1.1995	Tracheostomy tube	Łódź, ICU	Suspected pneumonia	G	7.6, 8.2	
L-902	*K. pneumoniae*	7.3.1995	Tracheostomy tube	Łódź, ICU	Suspected pneumonia	H	7.6, 8.2	
L-867	*E. coli*	30.4.1995	Tracheostomy tube	Łódź, ICU	Suspected pneumonia		5.4, 6.0, >9.0	TEM-49

[a] Underlined pI values are those for β-lactamases which were also produced by transconjugants.
[b] UTI, urinary tract infection.
[c] ICU, intensive care unit.

TABLE 2. Antimicrobial susceptibilities of clinical isolates and transconjugants

Strain no. (pI of ESBL in R^+ strains)[a]	MIC (μg/ml)													
	Ampicillin	Piperacillin	Piperacillin + tazobactam	Cefotaxime	Cefotaxime + clavulanate	Cefotaxime + tazobactam	Ceftazidime	Ceftazidime + clavulanate	Ceftazidime + tazobactam	Aztreonam	Aztreonam + clavulanate	Aztreonam + tazobactam	Cefoxitin	Imipenem
K. pneumoniae														
L-267	>512	>512	8	32	0.25	0.25	128	0.5	0.5	128	0.125	0.25	16	0.125
L-372	>512	>512	8	8	0.125	0.125	64	0.5	0.5	64	0.25	0.25	4	0.125
L-380	>512	>512	8	8	0.125	0.125	64	0.5	0.5	64	0.25	0.25	4	0.125
L-491	>512	>512	8	16	0.25	0.25	128	0.5	0.5	128	0.25	0.25	8	0.25
L-577	>512	>512	8	8	0.125	0.125	128	0.5	0.5	128	0.25	0.25	8	0.125
L-439	>512	>512	4	32	0.25	0.25	128	0.5	0.5	128	0.125	0.25	2	0.25
L-414	>512	>512	4	16	0.06	0.06	128	0.5	0.5	256	0.125	0.5	2	0.125
L-251	>512	>512	8	32	0.06	0.5	512	1	2	512	0.125	0.5	8	0.125
L-95	>512	>512	16	32	0.125	0.5	512	2	8	512	0.125	4	8	0.125
L-352	>512	512	4	16	0.125	0.5	256	1	1	256	0.06	0.25	4	0.25
L-902	>512	>512	16	64	0.125	0.5	512	2	8	512	0.25	2	8	0.125
E. coli L-867	>512	>512	16	64	16	4	64	16	8	128	8	8	64	0.125
Transconjugants														
R^+ L-267 (6.0)	>512	256	0.5	8	≤0.03	≤0.03	32	0.125	0.125	32	0.06	0.06	2	0.25
R^+ L-372 (6.0)	>512	256	0.5	8	≤0.03	0.06	16	0.125	0.125	32	0.06	0.06	2	0.25
R^+ L-380 (6.0)	>512	512	0.5	16	≤0.03	≤0.03	32	0.125	0.125	64	0.06	0.06	2	0.25
R^+ L-491 (6.0)	>512	256	0.5	8	≤0.03	≤0.03	16	0.125	0.125	32	0.06	0.06	4	0.25
R^+ L-577 (6.0)	>512	256	0.5	8	≤0.03	≤0.03	16	0.125	0.125	32	0.06	0.06	2	0.25
R^+ L-439 (8.2)	256	32	1	2	≤0.03	≤0.03	16	0.06	0.125	32	0.06	0.06	2	0.25
R^+ L-414 (8.2)	256	32	1	2	≤0.03	≤0.03	16	0.06	0.125	32	≤0.03	≤0.03	2	0.25
R^+ L-251 (8.2)	512	64	0.5	4	≤0.03	≤0.03	32	0.06	0.125	64	0.06	0.06	2	0.25
R^+ L-867 (6.0)	>512	128	0.5	2	≤0.03	≤0.03	16	0.06	0.125	32	0.06	≤0.03	2	0.25
E. coli A15 (recipient)	2	0.5	0.5	≤0.03	≤0.03	≤0.03	0.06	0.06	0.06	0.06	≤0.03	≤0.03	2	0.25

[a] R^+, transconjugants.

Susceptibility testing of clinical isolates. Results of the MIC analysis are presented in Table 2. All the clinical isolates were resistant to ampicillin and piperacillin (MICs, ≥512 μg/ml) and to ceftazidime and aztreonam (MIC range, 64 to 512 μg/ml). The MICs of cefotaxime were also raised; however, only two isolates (*K. pneumoniae* L-902 and *E. coli* L-867) were resistant to this antibiotic according to the criteria of the National Committee for Clinical Laboratory Standards (MIC, 64 μg/ml). *E. coli* L-867 was the only isolate resistant to cefoxitin (MIC, 64 μg/ml); all the isolates were susceptible to imipenem. Clavulanate and tazobactam restored the activities of the β-lactams against the isolates for all combinations tested.

IEF analysis of β-lactamases expressed by clinical isolates. The results of the IEF analysis of the β-lactamases are presented in Table 1. All *K. pneumoniae* isolates expressed β-lactamases with a pI of 7.6. The L-267 strain from Łódź and the group of four isolates from Rzeszów (isolates L-372, L-380, L-491, and L-577) produced enzymes with a pI of 6.0. Six strains from all three hospitals (strains L-439, L-414, L-251, L-95, L-352, and L-902) produced a β-lactamase with a pI of 8.2. Seven *K. pneumoniae* isolates were also found to express enzymes with pIs of or about 5.4. *E. coli* L-867 from Łódź expressed three β-lactamases with pIs of about 9.0, 6.0, and 5.4.

Resistance transfer experiment and susceptibility testing of recombinant strains. Transconjugants were obtained for all strains except *K. pneumoniae* L-95, L-352, and L-902. The results of the susceptibility testing (Table 2) have indicated that the MICs of ampicillin, piperacillin, cefotaxime, ceftazidime, and aztreonam for the transconjugants were higher than for the *E. coli* A15 recipient strain. As for the clinical isolates, the MICs of cefotaxime (2 to 16 μg/ml) were lower than those of ceftazidime (16 to 32 μg/ml) and aztreonam (32 to 64 μg/ml). β-Lactamase inhibitors reduced efficiently the MICs of the β-lactams used in the combinations. The cefoxitin resistance of *E. coli* L-867 was not transferred to the transconjugant strain.

IEF of transconjugant β-lactamases and bioassays for the detection of ceftazidime-hydrolyzing activity. Extracts from the *E. coli* transconjugant strains were subjected to IEF followed by assays for the detection of ceftazidime-hydrolyzing activity. Results of the analysis are presented in Table 1. The transconjugants of *K. pneumoniae* L-267, L-372, L-380, L-491, and L-577 and of *E. coli* L-867 produced β-lactamases with a pI of 6.0. Transconjugants of *K. pneumoniae* L-439, L-414, and L-251 expressed enzymes with a pI of 8.2. All these β-lactamases were confirmed to possess ceftazidime-hydrolyzing activity in subsequent bioassay experiments (data not shown).

PCR amplification of bla_{TEM} and bla_{SHV} genes. TEM-A and TEM-B primers (28) were used in reactions with plasmid preparations from transconjugants of *K. pneumoniae* L-267, L-372, L-380, L-491, and L-577 and *E. coli* L-867. PCR products of the expected size of about 1,100 bp, covering the entire coding sequence of a bla_{TEM} gene together with the promoter region, were detected in all isolates (data not shown). SHV-A and SHV-B primers, specific for bla_{SHV} sequences (7), were used for the PCR with plasmid DNA from transconjugants of *K.*

TABLE 3. Nucleotide sequences of bla_{TEM-47}, bla_{TEM-48}, and bla_{TEM-49} genes compared with those of bla_{TEM-1a}, bla_{TEM-1b}, bla_{TEM-2}, and some bla_{TEM-2}-related genes

Nucleotide[a] (amino acid[b]) position	Nucleotide (amino acid)[c]								
	bla_{TEM-1a}[d]	bla_{TEM-1b}[e]	bla_{TEM-2}[e]	bla_{TEM-4}[f]	bla_{TEM-9}[g]	bla_{TEM-25}[h]	bla_{TEM-48}	bla_{TEM-49}	bla_{TEM-47}
226	C	T							T
263 (21)	C (Leu)			T (Phe)	T (Phe)	T (Phe)	T (Phe)	T (Phe)	
317 (39)	C (Gln)		A (Lys)						
346	A		G	G	G	G	G	G	
436	C	T	T	T	T	T	T	T	T
512 (104)	G (Glu)			A (Lys)	A (Lys)				
604	G	T							
682	T		C	C	C	C	C	C	C
692 (164)	C (Arg)				A (Ser)				
914 (238)	G (Gly)			A (Ser)		A (Ser)	A (Ser)	A (Ser)	A (Ser)
917 (240)	G (Glu)						A (Lys)	A (Lys)	A (Lys)
925	G		A	A	A	A	A	A	A
990 (265)	C (Thr)			T (Met)	T (Met)	T (Met)	T (Met)	T (Met)	T (Met)
998 (268)	A (Ser)							G (Gly)	

[a] Nucleotide positions are numbered according to Sutcliffe (38).
[b] Amino acid positions are numbered according to Ambler et al. (1). Amino acid numbers are indicated only for positions in which substitutions are observed.
[c] Only mutational changes with respect to the TEM-1 β-lactamase and/or bla_{TEM-1a} gene sequences are listed.
[d] Data are from a previous report (38).
[e] Data are from previous reports (16, 17).
[f] Data are from a previous report (37).
[g] Data are from a previous report (28).
[h] Data are from a previous report (15).

pneumoniae L-439, L-414, and L-251. PCR products of the expected size of about 300 bp, representing the region of the bla_{SHV} genes in which some of mutations distinguishing particular SHV variants occur (7), were found in all isolates (data not shown).

Sequencing of TEM and SHV PCR products. The sequences of three different bla_{TEM} genes, encoding three different enzymes, were obtained for the isolates expressing β-lactamases with a pI of 6.0. The deduced amino acid sequences were compared with the amino acid sequence of the TEM-1 β-lactamase, and the nucleotide sequences of the coding regions were compared with the sequences of the bla_{TEM-1a} (38) and bla_{TEM-1b} and bla_{TEM-2} (16, 17) genes. Results of this analysis are presented in Table 3. All three β-lactamases represent new TEM variants designated TEM-47 (from *K. pneumoniae* L-267 from Łódź), TEM-48 (from *K. pneumoniae* L-372, L-380, L-491, and L-577 from Rzeszów), and TEM-49 (from *E. coli* L-867 from Łódź) (Table 1). All three β-lactamases share a triplet of amino acid substitutions: Gly238Ser (G914→A), Glu240Lys (G917→A), and Thr265Met (C990→T). Both TEM-48 and TEM-49 also have the Leu21Phe (C263→T) substitution, and TEM-49 alone also has the Ser268Gly substitution (A998→G). All three genes share three silent mutations with respect to the bla_{TEM-1a} sequence: C436→T, T682→C, and G925→A. The bla_{TEM-47} gene alone also has the C226→T mutation, whereas the bla_{TEM-48} and bla_{TEM-49} genes also possess the A346→G mutation.

Identical sequences of the region of about 210 bp of the bla_{SHV} gene were obtained for *K. pneumoniae* L-251, L-414, and L-439. This region corresponds to part of an SHV protein sequence located between Ala184 and Asn255 (1, 2). Analysis of the sequences has revealed that in all three strains parts of the genes coding for SHV ESBLs containing two substitutions (Gly238Ser and Glu240Lys) with respect to the sequence for the SHV-1 enzyme (2) were amplified.

DISCUSSION

Three different TEM ESBLs demonstrating new combinations of amino acid substitutions were identified during the analysis of 12 clinical isolates of the family *Enterobacteriaceae*. TEM-47, characterized by Gly238Ser, Glu240Lys, and Thr265 Met mutations, was found in a single *K. pneumoniae* strain from the hospital in Łódź. TEM-48, which also has the Leu21 Phe substitution, was produced by the group of four *K. pneumoniae* isolates from the hospital in Rzeszów. All of these isolates (two isolates were isolated from the same patient) had identical RAPD patterns, as determined by RAPD analysis with two different primers, which could indicate the spread of the TEM-48-producing strain in the pediatric ward of the hospital at the beginning of 1995. TEM-49 differs from TEM-48 by one more mutation, Ser268Gly, and was expressed by the single *E. coli* isolate from the hospital in Łódź.

The three amino acid substitutions which are shared by all TEM derivatives reported here have been well characterized in a series of studies. The Gly238Ser mutation alone characterizes SHV-2, the first ESBL ever described (3, 24, 25), and to date this mutation has been found in all SHV ESBLs and in several TEM variants (e.g., TEM-3, TEM-4, TEM-8, and TEM-25) (26). It is known that the mutation enlarges the substrate binding cavities of the enzymes, enabling them to interact with β-lactams having oxyimino side chains (20, 26). The Glu240Lys substitution characterizes three SHV mutants (SHV-4, SHV-5, and SHV-7) and some TEM mutants (e.g., TEM-5, TEM-10, TEM-24, and TEM-27) (26). The lysine residue binds to the carboxyl groups present in side chains of ceftazidime and aztreonam and efficiently raises the hydrolyzing activities of these enzymes toward these compounds (20, 26). The emergence of this mutation is linked to the selective pressure from the use of ceftazidime (10, 34). The simultaneous presence of Gly238Ser and Glu240Lys mutations is common for SHV enzymes (26), but interestingly, it is very rare within the TEM family and to date has been found only in the

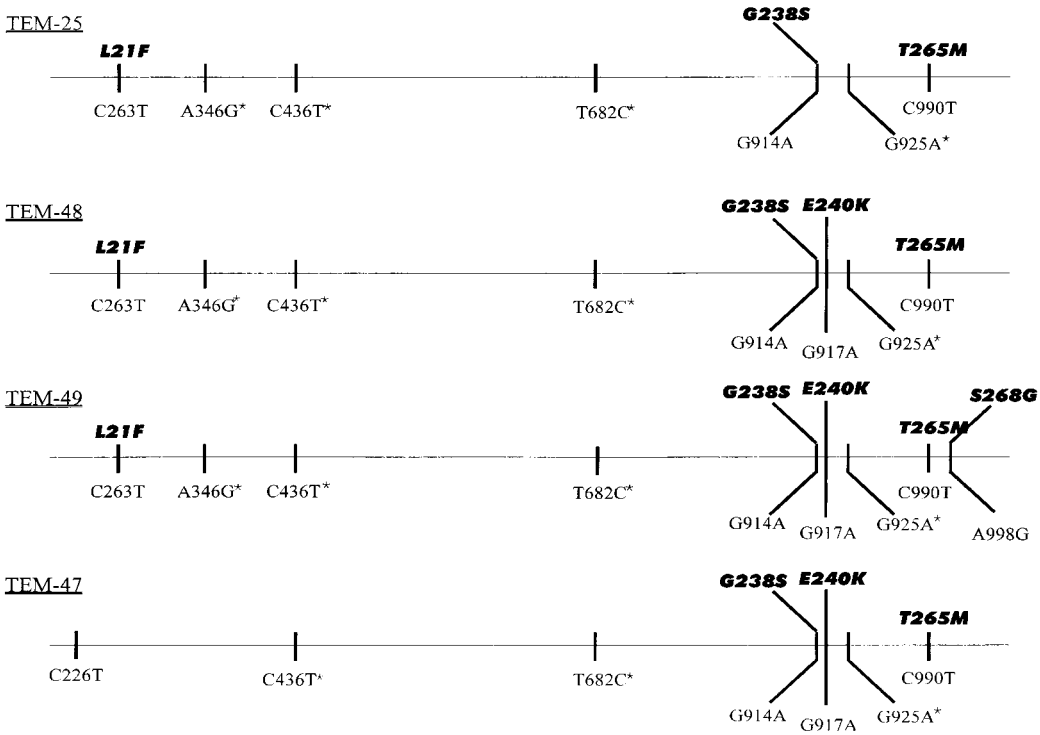

FIG. 1. Schematic representation of genes coding for TEM-47, TEM-48, TEM-49, and TEM-25 (15). All mutations found in the sequences of the genes described here in comparison to the sequence of the $bla_{\text{TEM-1a}}$ (38) gene are indicated and described below the lines representing genes (see also Table 3). Silent mutations characteristic of $bla_{\text{TEM-2}}$-related genes (16, 17) are marked by asterisks. It should be noted here that the C436T mutation is present also in the $bla_{\text{TEM-1b}}$ gene (16, 17). Amino acid substitutions caused by some of the mutations are presented above the lines in boldface type. Nucleotide numbering is according to Sutcliffe (38), and amino acid numbering is according to Ambler et al. (1).

TEM-42 variant (30). Among the collection of all possible amino acid combinations of the region of the TEM-1 β-lactamase from positions 237 to 240, the double Gly238Ser-Glu240 Lys mutant was selected as being optimal for ceftazidime hydrolysis (14). The Thr265Met substitution was found in several TEM variants (TEM-4, TEM-9, TEM-13, TEM-25, and TEM-27) (26). Experiments with the TEM-4 enzyme (37), the TEM-13 enzyme (21), the TEM-1 artificial mutant with the Gly238Ser-Glu240Lys-Thr265Met mutations (which is therefore identical to the TEM-47 enzyme) (19), and the mutant with the artificial single Thr265Met mutation (9) have revealed a lack of any effect of the Thr265Met mutation on β-lactamase activity.

The Leu21Phe mutation within the signal sequence is present in three TEM variants (TEM-4, TEM-9, and TEM-25) (15, 28, 37) and was also reported to influence neither the binding nor the hydrolysis of β-lactams (37). This is confirmed by the comparison of MIC data obtained for transconjugants producing the TEM-47 and TEM-48 enzymes. The Ser268Gly mutation observed within the TEM-49 sequence has not been reported to date, and its effect, if any, on β-lactamase activity remains unclear. Comparison of the MICs for transconjugants expressing TEM-48 and TEM-49 suggests the neutrality of this substitution.

The distribution of mutations within the deduced amino acid sequences of TEM-47, TEM-48 and TEM-49 could suggest that all these β-lactamases are very closely related and that TEM-48 and TEM-49 might have arisen by a sequential accumulation of substitutions starting from TEM-47. Convincing data on the evolution of one ESBL from another (TEM-12 and TEM-10) has already been described (10). Because the analyzed enzymes lack the Gln39Lys mutation that is characteristic of the TEM-2 β-lactamase (16, 17), the TEM-1 (38) enzyme might be the indirect progenitor of the group. However, comparison of the nucleotide sequences of the amplified $bla_{\text{TEM-47}}$, $bla_{\text{TEM-48}}$, and $bla_{\text{TEM-49}}$ genes and other bla_{TEM} genes (Table 3; Fig. 1) has revealed a much more complex view of their origins and relationships.

The sequences of the $bla_{\text{TEM-48}}$ and $bla_{\text{TEM-49}}$ genes differ by only one mutation, A→G, at position 998 (Ser268Gly substitution in the TEM-49 sequence). They share all four other substitutions and all four silent mutations (A346→G, C436→T, T682→C, and G925→A) present in their sequences in comparison with the $bla_{\text{TEM-1a}}$ nucleotide sequence. This makes likely the hypothesis that the described $bla_{\text{TEM-49}}$ gene has evolved from the $bla_{\text{TEM-48}}$ gene.

The four silent mutations observed in both genes are characteristic of the $bla_{\text{TEM-2}}$ gene but not the known $bla_{\text{TEM-1}}$ variants, even if the C436→T mutation also occurs in the $bla_{\text{TEM-1b}}$ gene (16, 17, 38). Hence, the genes coding for TEM-48 and TEM-49 β-lactamases seem to be more similar to $bla_{\text{TEM-2}}$ than to either variant of the $bla_{\text{TEM-1}}$ gene, indicating that they may share with the $bla_{\text{TEM-2}}$ gene a hypothetical common ancestor which may have possessed only the group of four silent mutations mentioned above. The same set of silent mutations together with the lack of the C317→A (Gln39Lys) substitution has already been reported for the $bla_{\text{TEM-4}}$ (37), $bla_{\text{TEM-9}}$ (28), $bla_{\text{TEM-12b}}$ (CAZ-3) (15, 18), and $bla_{\text{TEM-25}}$ (CTX-2) (15) genes, and their relationship to $bla_{\text{TEM-2}}$ has been postulated.

In fact, the $bla_{\text{TEM-48}}$ gene differs from the described $bla_{\text{TEM-25}}$ gene (15) by only one substitution, G917→A

(Glu240Lys), which is known to be one of the more efficiently selected mutations under the pressure of ceftazidime use (10, 34). This makes the bla_{TEM-25} gene a good candidate for being a direct default sequence for the $bla_{TEM-48} \rightarrow bla_{TEM-49}$ evolution. If one considers that the TEM-47 β-lactamase is the progenitor of TEM-48, one would have to postulate the second selection event of the Leu21Phe (C263→T) substitution (present also in TEM-4, TEM-9, and TEM-25) in the evolution of TEM-2-related β-lactamases. This would not be very likely, assuming that this mutation is probably neutral (28, 37). Other clues prompting us to reject the hypothesis that the TEM-47 enzyme is the direct ancestor of TEM-48 come from the analysis of the nucleotide sequence of the bla_{TEM-47} gene.

Of the four bla_{TEM-2}-specific synonymous mutations discussed here, the bla_{TEM-47} gene lacks the A346→G mutation but it possesses the C226→T silent mutation which was reported in the bla_{TEM-1b} sequence (17). Therefore, by also considering its lack of the C263→T (Leu21Phe) substitution, it is less similar to the gene encoding TEM-48 than the bla_{TEM-25} gene and could not have been the default sequence for the evolution of bla_{TEM-48}. However, all three differences are placed in the 5′ part of the gene (ca. 140 bp of the coding region), and downstream from nucleotide position 346, the bla_{TEM-47} and bla_{TEM-48} genes have identical sequences containing three substitutions (G914→A [Gly238Ser], G917→A [Glu240Lys], and C990→T [Thr265Met]) and the remaining three bla_{TEM-2}-like silent mutations (C436→T, T682→C, and G925→A). This could, in spite of differences, suggest the possibility of the close relatedness between the identified sequences encoding TEM-47 and TEM-48. It can be postulated that the bla_{TEM-47} gene has arisen from the bla_{TEM-48} gene by a recombination event with a bla_{TEM-1b}-related gene leading to the exchange of the 5′ ends of the genes.

The six remaining *K. pneumoniae* isolates examined, all with different RAPD patterns, appeared to produce SHV-5-like ESBLs. A β-lactamase band comigrating with the SHV-5 standard (pI 8.2) was detected in extracts of all these strains. ESBLs from three strains (strains L-251 and L-414 from Łódź and strain L-439 from Rzeszów) were studied to the sequence level. Partial gene sequences covering the region in which some of the critical mutations in the SHV family occur were identical to the bla_{SHV-5} (7) and bla_{SHV-12} (33) gene sequences and indicated the presence of Gly238Ser and Glu240Lys substitutions in the deduced protein sequences. The same sequence within the reported region also characterizes the SHV-7 (11) β-lactamase, but additional mutations in other parts of the enzyme make it easily distinguishable from SHV-5 by its pI value (11). Three other strains (strain L-95 from Warsaw and strains L-352 and L-902 from Łódź) did not give recombinants in conjugation experiments, and our supposition about their production of SHV-5-like enzymes is based only on the analysis of β-lactamase patterns. The failure of the resistance transfer experiment might have been due to a chromosomal location of ESBL genes or, more likely, to the presence of the ESBL genes on nontransmissible plasmids. The SHV-5 and SHV-5-related β-lactamases appear to belong to the predominant types of ESBLs and to date have been identified in several countries (5, 27, 31, 33, 36, 40, 41). Our data showing the presence of bla_{SHV-5}-type genes in very small groups of isolates from three randomly chosen Polish hospitals may suggest that SHV-5-like enzymes are also widespread in Poland.

The growing use of a new generation of β-lactams in Central Europe in recent years creates a high risk for the very efficient selection of strains of the family *Enterobacteriaceae* harboring β-lactamases with extended-spectrum activity. Our observations indicate that this is already a serious problem in Polish hospitals.

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