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Evolution of TEM-Type Extended-Spectrum β-Lactamases in Clinical *Enterobacteriaceae* Strains in Poland

Anna Baraniak, Janusz Fiett, Agnieszka Mrówka, Jarosław Walory, Waleria Hryniewicz, and Marek Gniadkowski*

National Institute of Public Health, Chełmska 30/34, 00-725 Warsaw, Poland

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Seventeen extended-spectrum β-lactamase (ESBL)-producing isolates of the family *Enterobacteriaceae* recovered from 1998 to 2000 in hospitals of five different cities in Poland were analyzed. They expressed several TEM-type ESBLs, TEM-4, TEM-29, TEM-85, TEM-86, TEM-93, and TEM-94. TEM-85 (L21F, R164S, E240K, T265M), TEM-86 (L21F, R164S, A237T, E240K, T265M), TEM-93 (M182T, G238S, E240K), and TEM-94 (L21F, E104K, M182T, G238S, T265M) were identified for the first time. Including the enzymes described earlier, TEM-47, TEM-48, TEM-49, and TEM-68, the group of known ESBLs of the TEM family produced by enterobacteria in Polish hospitals has increased to 10 variants. Comparative sequence analysis of the genes coding for all these β-lactamases revealed a view of their possible evolution, which, apart from the gradual acquisition of various mutations, could also have involved recombination events. Two different $bla_{\text{TEM-1}}$ gene alleles were precursors of the ESBL genes: $bla_{\text{TEM-1A}}$, which was the ancestor of $bla_{\text{TEM-93}}$, and $bla_{\text{TEM-1F}}$, from which all the remaining genes originated. The evolution of the $bla_{\text{TEM-1F}}$-related genes most probably consisted of three major separate lineages, one of which, including $bla_{\text{TEM-4}}$, $bla_{\text{TEM-47}}$, $bla_{\text{TEM-48}}$, $bla_{\text{TEM-49}}$, $bla_{\text{TEM-68}}$, and $bla_{\text{TEM-94}}$, was highly structured itself and could have been initiated by the $bla_{\text{TEM-25}}$ gene, identified exclusively in France so far. Plasmid fingerprinting analysis revealed a high degree of diversity of plasmids carrying related bla_{TEM} genes, which suggested either the intense diversification or transposition of bla_{TEM} genes between different plasmids or some contribution of convergent evolution. The results of this study clearly demonstrate that the environment of Polish hospitals has been highly favorable for the rapid evolution of ESBLs.

The β-lactamases of gram-negative rods are responsible for the most rapidly evolving mechanisms of resistance in pathogenic bacteria under the selection pressure of antibiotic use (17, 28, 30). One of the major aspects of this evolution is the accumulation of mutations, which result, for example, in modifications of the enzymes' catalytic efficiencies, substrate spectra, and susceptibilities to inhibitors (25, 30). The gradual acquisition of mutations has recently led to a dramatic increase in the number of β-lactamase variants observed, which is well exemplified by some families of the Ambler class A enzymes (1), namely, TEM, SHV, and CTX-M (www.lahey.org/studies/webt.htm). They include the vast majority of extended-spectrum β-lactamases (ESBLs) that are the main source of resistance of gram-negative bacteria to oxyimino-β-lactams (8, 28). A number of reports delivered a large amount of evidence on the direct emergence of one enzyme variant from another within these families (3, 9, 11, 32); however, wider views of the possible β-lactamase evolution in general (16) or in hospitals of a given region (13) have been proposed in only a few papers.

The TEM family of ESBLs constitutes the largest and widely disseminated group of these enzymes. Their evolutionary precursors are the TEM-1 and TEM-2 penicillinases (8, 28, 30), of which TEM-1 is encoded by a series of gene alleles, $bla_{\text{TEM-1A}}$ to $bla_{\text{TEM-1F}}$, which differ from each other by specific silent mutations. Each of these genes could initiate a separate evolutionary lineage of mutant derivatives (27). In previous reports we described four TEM ESBLs, TEM-47, TEM-48, TEM-49, and TEM-68, which have been observed exclusively in Poland so far. The $bla_{\text{TEM-48}}$ gene could be a direct precursor of the remaining ones, of which $bla_{\text{TEM-49}}$ could emerge due a point mutation and $bla_{\text{TEM-47}}$ could be due to crossing over between $bla_{\text{TEM-48}}$ and a $bla_{\text{TEM-1B}}$-type gene (19). Then, $bla_{\text{TEM-68}}$ most probably arose from a single mutation in $bla_{\text{TEM-47}}$ (15). It was also proposed that $bla_{\text{TEM-48}}$ could have evolved by a point mutation from the $bla_{\text{TEM-25}}$ gene (19), which had been identified in France (10). The specific set of silent mutations with respect to $bla_{\text{TEM-1A}}$ (27), modified in $bla_{\text{TEM-47}}$ and $bla_{\text{TEM-68}}$ by the putative crossing over, indicated that all these genes were descendants of $bla_{\text{TEM-1F}}$ (15, 19). The study reported here has been a continuation of efforts aimed at monitoring the spread and evolution of TEM ESBLs in enterobacteria in Polish hospitals.

MATERIALS AND METHODS

Clinical isolates. The analysis was carried out with 17 ESBL-producing isolates of the family *Enterobacteriaceae* (*Klebsiella pneumoniae*, $n = 9$; *Klebsiella oxytoca*, $n = 1$; *Escherichia coli*, $n = 7$), recovered in five hospitals in different Polish cities (Table 1). Fourteen isolates from Gdańsk, Kraków, and Suwałki were collected during a 1998 survey of ESBL types in enterobacteria in Poland (M. Gniadkowski, A. Baraniak, J. Fiett, and W. Hryniewicz, unpublished results) and were selected for the study as putative ESBL producers of the TEM family on the basis of the preliminary analysis of their β-lactamase contents (by isoelectric focusing). The three remaining isolates, from Częstochowa and Bielsko-Biała (*K. pneumoniae* CZ9455/99 and CZ9459/99 and *K. oxytoca* BB1753/00), were identified in 1999 and 2000 and were sent to the National Institute of Public Health in Warsaw as a result of other multicenter studies on antimicrobial resistance. They

* Corresponding author. Mailing address: National Institute of Public Health, ul. Chełmska 30/34, 00-725 Warsaw, Poland. Phone: 48 22-851 43 88. Fax: 48 22-841 29 49. E-mail: gniadkow@cls.edu.pl.

TABLE 1. Clinical isolates, β-lactamases in the isolates and transconjugants, ESBLs identified by bioassay and DNA sequencing, and typing data

Isolate	Date of isolation (mo.yr)	City of isolation	pI(s) of β-lactamases[a]	Bioassay[b]	ESBL sequence	RAPD type[c]	PFGE type	Plasmid fingerprint
K. pneumoniae SU2946/98	03.98	Suwałki	7.6, 5.5	5.5 CAZ-CTX	TEM-86	A	ND[d]	E1
K. pneumoniae SU2949/98	03.98	Suwałki	7.6, 5.5	5.5 CAZ-CTX	TEM-85	A	ND	ND
K. pneumoniae SU3220/98	04.98	Suwałki	7.6, 5.5	5.5 CAZ-CTX	TEM-85	A	ND	E1
K. pneumoniae SU3519/98	05.98	Suwałki	7.6, 5.5	5.5 CAZ-CTX	TEM-85	A	ND	E1
E. coli SU2947/98	03.98	Suwałki	7.4, 5.5	5.5 CAZ-CTX	TEM-29	a	ND	F
E. coli SU3408/98	05.98	Suwałki	5.5	5.5 CAZ-CTX	TEM-85	b	ND	E2
K. pneumoniae GD3658/98	03.98	Gdańsk	7.6, 6.0	6.0 CTX-CAZ	TEM-47	B	e	A11
K. pneumoniae GD3661/98	04.98	Gdańsk	7.6, 6.0	6.0 CTX-CAZ	TEM-47	C	f	A10
K. pneumoniae GD3670/98	06.98	Gdańsk	6.0	6.0 CTX-CAZ	TEM-47	D	g	A9
E. coli GD3667/98	06.98	Gdańsk	6.0	6.0 CTX-CAZ	TEM-47	d	ND	A8
E. coli GD3657/98	03.98	Gdańsk	6.0	6.0 CTX-CAZ	TEM-94	c	ND	D
E. coli GD3668/98	06.98	Gdańsk	6.0	6.0 CTX-CAZ	TEM-94	c	ND	D
E. coli KR3445/98	03.98	Kraków	6.0	6.0 CTX-CAZ	TEM-93	e	ND	G
E. coli KR3447/98	04.98	Kraków	6.0	6.0 CTX-CAZ	TEM-93	f	ND	G
K. pneumoniae CZ9455/99[e]	10.99	Częstochowa	8.4, 7.6, 6.0, 5.4	6.0 CTX-CAZ 8.4 CTX	TEM-4[f] CTX-M-3	E	ND	C
K. pneumoniae CZ9459/99[e]	10.99	Częstochowa	8.4, 7.6, 6.0, 5.4	6.0 CTX-CAZ 8.4 CTX	TEM-4[f] CTX-M-3	E	ND	C
K. oxytoca BB1753/00	04.00	Bielsko-Biała	8.4, 6.0, 5.4	6.0 CTX-CAZ 8.4 CTX	TEM-4[f] CTX-M-3	ND	ND	C
K. pneumoniae WR3144/98[g]	01.96	Wrocław	7.6, 6.0	6.0 CTX-CAZ	TEM-47	G	a	A1
K. pneumoniae WR3159/98[g]	04.96	Wrocław	7.6, 6.0	6.0 CTX-CAZ	TEM-47	F	b2	A2
K. pneumoniae WR3162/98[g]	06.96	Wrocław	7.6, 6.0	6.0 CTX-CAZ	TEM-47	H	d	A3
K. pneumoniae LD L-267[h]	01.95	Łódź	7.6, 6.0	6.0 CTX-CAZ	TEM-47	F	b2	A5
K. pneumoniae RZ L-372[h]	01.95	Rzeszów	7.6, 6.0, 5.4	6.0 CTX-CAZ	TEM-48[f]	I	h	B
E. coli LD L-867[h]	04.95	Łódź	9.0, 6.0, 5.4	6.0 CTX-CAZ	TEM-49[f]	ND	ND	ND
K. pneumoniae WA1027/96[i]	03.96	Warsaw	7.6, 6.0	6.0 CTX-CAZ	TEM-47	F	b3	A6
K. pneumoniae WA1099/96[i]	04.96	Warsaw	7.6, 6.0	6.0 CTX-CAZ	TEM-47	F	b4	A6
K. pneumoniae WA1592/96[i]	05.96	Warsaw	7.6, 6.0	6.0 CTX-CAZ	TEM-47	F	b5	A7
K. pneumoniae WR3151/98[g]	06.96	Wrocław	7.6, 5.7	5.7 CTX-CAZ	TEM-68	G	a	A4

[a] Underlined pI values are those for β-lactamases which were also produced by the transconjugants.
[b] CTX-CAZ and CTX, an enzyme with a given pI hydrolyzed both cefotaxime (CTX) and ceftazidime (CAZ) or only cefotaxime, respectively, under the experimental conditions.
[c] Capital letters designate the RAPD types of *K. pneumoniae*; lowercase letters designate the RAPD types of *E. coli* isolates.
[d] ND, not determined; in the case of plasmid fingerprinting, the restriction patterns of these plasmids were difficult to interpret.
[e] The β-lactamase content, mating, bioassay, and CTX-M-3 sequence data for these isolates were published previously (4).
[f] Detection of the bla_{TEM} ESBL gene was performed with plasmid DNA purified from the transconjugant strain.
[g] Data for these isolates were published previously (15).
[h] Data for these isolates were published previously (19).
[i] Data for these isolates were published previously (18).

were classified as possible TEM- and CTX-M-type double ESBL producers, and *K. pneumoniae* CZ9455/99 and CZ9459/99 were partially analyzed in previous work (4) with CTX-M-3-producing organisms in Poland. The isolates were recovered from various specimens, mostly urine, and were identified with the ATB ID32E test (bioMérieux, Charbonnieres-les-Bains, France). ESBL production was indicated by positive results of the double-disk synergy test (24).

Ten additional enterobacterial isolates, characterized in earlier studies, were included in the analysis for comparative purposes (Table 1). The nine *K. pneumoniae* and one *E. coli* isolates, identified in 1995 and 1996 in four other Polish cities (Łódź, Rzeszów, Warsaw, and Wrocław), produced four ESBL variants of the TEM family, TEM-47, TEM-48, TEM-49, and TEM-68 (15, 18, 19).

Antimicrobial susceptibility testing. The MICs of various β-lactam antibiotics were evaluated by the agar dilution method according to the guidelines of the NCCLS (31). The following compounds were used: ampicillin and cefotaxime (Polfa Tarchomin, Warsaw, Poland), aztreonam (Bristol-Myers Squibb, New Brunswick, N.J.), cefoxitin (Sigma Chemical Company, St. Louis, Mo.), ceftazidime (GlaxoSmithKline, Stevenage, United Kingdom), cephalothin (Sigma Chemical Company), lithium clavulanate (GlaxoSmithKline, Betchworth, United Kingdom), imipenem (Merck Sharp & Dohme, Rahway, N.J.), and piperacillin and tazobactam (Wyeth, Pearl River, N.Y.). In 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.

Mating. The isolates were subjected to ceftazidime or cefotaxime resistance transfer experiments as described previously (19), with *E. coli* A15, which is resistant to rifampin, used as the recipient strain. Transconjugants were selected on MacConkey agar (Oxoid, Basingstoke, United Kingdom) supplemented with 2 μg/ml ceftazidime or cefotaxime and 128 μg/ml rifampin (Polfa Tarchomin).

IEF of β-lactamases and bioassay for oxyiminocephalosporin-hydrolyzing activities. The β-lactamase contents of the isolates and their transconjugants were analyzed by isoelectric focusing (IEF), as described by Bauernfeind et al. (6), with a model 111 Mini IEF Cell (Bio-Rad, Hercules, Calif.). After IEF, the cefotaxime- and ceftazidime-hydrolyzing activities were assigned to particular β-lactamases by bioassay, as described by Bauernfeind et al. (6).

PCR detection and sequencing of bla_{TEM} genes. Total bacterial DNA was purified with a Genomic DNA Prep Plus kit (A & A Biotechnology, Gdańsk, Poland). The entire bla_{TEM} genes were amplified by PCR with primers TEM-A and TEM-B (29) under the conditions described before (19). The amplicons were purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and directly sequenced with an ABI PRISM 310 sequencer (Applied Biosystems, Foster City, Calif.). Sequencing of the whole genes was performed with primers TEM-A, TEM-B, TEM-C, TEM-D, and TEM-E, whereas only the promoter regions were sequenced with primers TEM-A and TEM-G (29).

Cloning of bla_{TEM} genes. The bla_{TEM-85}, bla_{TEM-86}, bla_{TEM-93}, and bla_{TEM-94} genes were amplified by PCR with primers TEM-A/EcoRI and TEM-B/BamHI,

as described previously (15). The resulting amplicons were cut with EcoRI and BamHI (MBI Fermentas, Vilnius, Lithuania) and cloned into the plasmid vector pGB2 (14). *E. coli* DH5α transformants were selected on tryptic soy agar (Oxoid) supplemented with 2 μg/ml ceftazidime and 30 μg/ml streptomycin (Polfa Tarchomin).

RAPD and PFGE typing. Randomly amplified polymorphic DNA (RAPD) analysis was carried out separately with two primers, RAPD-7 and RAPD-1283 (33), as reported previously (19). Pulsed-field gel electrophoresis (PFGE) analysis was performed as described by Struelens et al. (35) with the XbaI restriction enzyme (MBI Fermentas) and a CHEF DRIII PFGE system (Bio-Rad). The PFGE results were interpreted as described by Tenover et al. (37).

Plasmid fingerprinting. Plasmid DNA was purified with a Plasmid Midi kit (QIAGEN), according to the recommendations of the manufacturer. For the fingerprinting analysis, plasmids were digested with the PstI restriction enzyme (MBI Fermentas) and electrophoresed in 1% agarose gels (SeaKem; Cambrex, Rockland, Maine).

Nucleotide sequence accession numbers. The bla_{TEM} sequences analyzed in this work appear in the EMBL database under the following accession numbers: bla_{TEM-29}, AJ277416; bla_{TEM-85}, AJ277414; bla_{TEM-86}, AJ277415; bla_{TEM-93}, AJ318093; and bla_{TEM-94}, AJ318094.

RESULTS

β-Lactamases of the clinical isolates. The IEF analysis demonstrated a variety of β-lactamases in the isolates studied (Table 1). The more common types of β-lactamases were enzymes with a pI of 5.5, expressed by four *K. pneumoniae* and two *E. coli* isolates from the hospital in Suwałki, and β-lactamases with a pI of 6.0, observed in extracts of all the remaining isolates. Additionally, β-lactamases with a pI of 7.6 were produced by almost all *K. pneumoniae* isolates, and enzymes with pIs of 8.4 and 5.4 were expressed by *K. pneumoniae* CZ9455/99 and CZ9459/99 and *K. oxytoca* BB1753/00. The extract of *E. coli* SU2947/98 also contained a pI 7.4 β-lactamase. The bioassay analysis (Table 1) revealed that only the pI 5.5, 6.0, and 8.4 β-lactamases hydrolyzed oxyiminocephalosporins under the experimental conditions used, which indicated their ESBL activities. Whereas the pI 5.5 and 6.0 enzymes hydrolyzed both ceftazidime and cefotaxime, the pI 8.4 β-lactamases only hydrolyzed cefotaxime. Further analysis of *K. pneumoniae* CZ9455/99 and CZ9459/99 revealed that the pI 8.4 enzyme was CTX-M-3 (4), and the same was found for *K. oxytoca* BB1753/00 (data not shown).

Mating and β-lactamases of the transconjugants. The results for the transconjugants are shown in Table 1. Of the six isolates with pI 5.5 ESBLs, only *E. coli* SU3408/98 produced transconjugants, which expressed the pI 5.5 enzyme as well. Of the 11 isolates with pI 6.0 ESBLs, transconjugants were obtained for 9 isolates (including all those with the additional CTX-M-3 enzyme), and they all exclusively expressed the pI 6.0 enzymes. The results for *K. pneumoniae* CZ9455/99 and CZ9459/99 have been reported previously (4).

PCR detection and sequences of the bla_{TEM}-coding regions. The total DNAs of the isolates were tested for the presence of bla_{TEM} genes. In the case of *K. pneumoniae* CZ9455/99 and CZ9459/99 and *K. oxytoca* BB1753/00, the DNAs of their transconjugants were used, since these isolates also produced a pI 5.4 β-lactamase, likely TEM-1. PCR with bla_{TEM}-specific primers yielded products of about 1 kb in all cases; the results obtained for *K. pneumoniae* CZ9455/99 and CZ9459/99 have already been published (4).

All the amplicons were sequenced, and the results are shown in Tables 1 and 2. Analysis of the deduced amino acid sequences and comparison of the sequences with those deposited in the TEM β-lactamase database (www.lahey.org/studies/webt.htm) revealed that the pI 5.5 TEM enzymes included three different variants, TEM-29, TEM-85, and TEM-86. TEM-29 had been identified in France before (2), whereas the last two β-lactamases were novel TEM ESBL variants. TEM-85 was characterized by L21F, R164S, E240K, and T265M amino acid substitutions with respect to the sequence of TEM-1 (36); and TEM-86 differed from TEM-85 only by a single additional substitution, A237T. Among the pI 6.0 enzymes, four different TEM variants were found; and these were TEM-4, TEM-47, TEM-93, and TEM-94. TEM-4 and TEM-47 had originally been described in France and Poland, respectively (19, 34), but TEM-93 and TEM-94 were identified for the first time. TEM-93 carried three substitutions, M182T, G238S, and E240K, compared to the sequence of TEM-1, whereas TEM-94 was characterized by the L21F, E104K, M182T, G238S, and T265M substitutions.

Apart from the mutations which caused amino acid substitutions, almost all the bla_{TEM} genes also contained silent mutations with respect to the sequence of bla_{TEM-1A} (Table 2) (36). The full set of four such mutations that is characteristic for the bla_{TEM-1F} gene (27), A346→G, C436→T, T682→C, and G925→A, was identified in bla_{TEM-4}, bla_{TEM-29}, bla_{TEM-85}, bla_{TEM-86}, and bla_{TEM-94}. This indicated that of the enzyme variants that had been observed before in France, TEM-4 was encoded by an identical gene (34), whereas TEM-29 was specified by another nucleotide sequence (2). The bla_{TEM-47} genes were identical to those described earlier in Poland (15, 18, 19), with the silent mutations C226→T, C436→T, T682→C, and G925→A. Only the bla_{TEM-93} gene did not contain any silent mutations compared to the sequence of bla_{TEM-1A} (36).

Promoter regions of the bla_{TEM} genes. In order to identify the promoters of the bla_{TEM} genes, the sequences located upstream from their coding regions were compared with that of the bla_{TEM-1A} gene, which is driven by the promoter *P3* (27). Moreover, the promoter sequences of the previously identified bla_{TEM-47}, bla_{TEM-48}, bla_{TEM-49}, and bla_{TEM-68} genes were determined for 10 selected isolates from earlier work (15, 18, 19). The results are shown in Table 2. The sequences of all but one of the promoter regions differed from that of *P3* only by the presence of a T instead of a C at position 32; therefore, they were identified as the double overlapping promoter *Pa*/*Pb* (27). The only exception was the promoter of bla_{TEM-49} from the *E. coli* L-867 isolate (19), whose sequence was identical to that of *P3*. Additionally, the sequences of the 5′ regions of all bla_{TEM-47} and bla_{TEM-68} genes differed from that of bla_{TEM-1A} by a G instead of an A at position 175.

Typing. The *E. coli* and *K. pneumoniae* isolates were typed by RAPD analysis, as were nine representative TEM-47-, TEM-48-, and TEM-68-producing *K. pneumoniae* isolates studied before (15, 18, 19). The results (Table 1) confirmed our previous observations of the similarity of the older TEM-47-producing *K. pneumoniae* isolates from Łódź, Warsaw, and Wrocław (RAPD type F) and of the other, older TEM-47 and TEM-68 producers from Wrocław (RAPD type G) (15, 18). The RAPD patterns of all of the newer *K. pneumoniae* isolates differed from those of the older ones; moreover, the only similarities among them were observed within groups from a single center. The four TEM-85- and TEM-86-producing *K.*

TABLE 2. Nucleotide sequences of bla_{TEM}-coding regions and their promoters

Nucleotide[a] (amino acid[b]) position	Nucleotide (amino acid)[c] in bla_{TEM}-coding regions and their promoters													
	bla_{TEM-1A}[d] (*P3*)	bla_{TEM-93} (*Pa/Pb*)	bla_{TEM-1F}[e] (*P4*)	bla_{TEM-1B}[f] (*P3*)	bla_{TEM-25}[g] (*?*)	bla_{TEM-4}[h] (*Pa/Pb*)	bla_{TEM-94} (*Pa/Pb*)	bla_{TEM-48}[i] (*Pa/Pb*)	bla_{TEM-49}[i] (*P3*)	bla_{TEM-47}[i] (*Pa/Pb*)	bla_{TEM-68}[j] (*Pa/Pb*)	bla_{TEM-29}[k] (*Pa/Pb*)	bla_{TEM-85} (*Pa/Pb*)	bla_{TEM-86} (*Pa/Pb*)
32	C	T			?	T	T	T		T	T	T	T	T
162	G		T		?									
175	A			G	?					G	G			
226	C			T						T	T			
263 (21)	C (Leu)				T (Phe)	T (Phe)	T (Phe)	T (Phe)	T (Phe)				T (Phe)	T (Phe)
346	A		G		G	G	G	G	G			G	G	G
436	C		T	T	T	T	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	C	C	C	C
692 (164)	C (Arg)												A (Ser)	A (Ser)
693 (164)	G (Arg)											A (His)		
747 (182)	T (Met)	C (Thr)					C (Thr)							
911 (237)	G (Ala)													A (Thr)
914 (238)	G (Gly)	A (Ser)			A (Ser)	A (Ser)	A (Ser)	A (Ser)	A (Ser)	A (Ser)	A (Ser)			
917 (240)	G (Glu)	A (Lys)						A (Lys)	A (Lys)	A (Lys)	A (Lys)		A (Lys)	A (Lys)
925	G		A		A	A	A	A	A	A	A	A	A	A
990 (265)	C (Thr)				T (Met)	T (Met)	T (Met)	T (Met)	T (Met)	T (Met)	T (Met)		T (Met)	T (Met)
998 (268)	A (Ser)								G (Gly)					
1020 (275)	G (Arg)										T (Leu)			

[a] Nucleotide positions are numbered according to Sutcliffe (36).
[b] Amino acid positions are numbered according to Ambler et al. (1). The numbers are indicated only for positions in which substitutions were observed.
[c] Only mutational changes with respect to the TEM-1 β-lactamase and/or bla_{TEM-1A} gene sequences are listed.
[d] Reference 36.
[e] Reference 27.
[f] Reference 21.
[g] Reference 10.
[h] Reference 34 and this work.
[i] Reference 19.
[j] Reference 15.
[k] Reference 2 and this work.

pneumoniae isolates from Suwałki were indistinguishable from each other, and this was the case for the two TEM-4 (and CTX-M-3)-producing *K. pneumoniae* isolates from Częstochowa. Similarly, the two TEM-94-producing *E. coli* isolates from Gdańsk were characterized by identical RAPD patterns.

The TEM-47-producing *K. pneumoniae* isolates from Gdańsk, together with the older TEM-47-, TEM-48-, and TEM-68-producing isolates of this species, were also typed by PFGE. The results (Table 1) were in concordance with the RAPD data and the partial earlier observations (15). The clonal structure of the group of older isolates consisted of four clones. The first of these included TEM-47-producing isolates from Warsaw, Łódź, and Wrocław (PFGE type b), whereas two others, with TEM-47 or TEM-68 or only TEM-47, were present only in Wrocław (types a and d, respectively). The TEM-48-producing *K. pneumoniae* isolate from Rzeszów (19) represented a separate clone (PFGE type h). The newer TEM-47-producing isolates from Gdańsk were not related to each other or to any of the clones identified before (PFGE types e, f and g).

Plasmid fingerprinting. Plasmid DNA was purified from the transconjugants or clinical isolates and subjected to fingerprinting analysis. Plasmids specific for the older isolates producing TEM-47, TEM-48, TEM-49, and TEM-68 (15, 18, 19) were also fingerprinted. The results are shown in Table 1. In general, the plasmids were highly diverse; however, some clear similarities in their PstI fingerprints could be observed. The four TEM-47-producing *K. pneumoniae* and *E. coli* isolates from Gdańsk contained large plasmids that were similar to each other and to plasmids carried by the older TEM-47- and TEM-68-producing isolates from Łódź, Warsaw, and Wrocław (fingerprints A1 to A11). Plasmids present in the three TEM-4-producing *K. pneumoniae* and *K. oxytoca* isolates from Częstochowa and Bielsko-Biała were indistinguishable from each other (fingerprint C), and this was also the case for the two TEM-94-producing *E. coli* isolates from Gdańsk (fingerprint D), TEM-85- or TEM-86-producing *K. pneumoniae* and *E. coli* isolates from Suwałki (fingerprints E1 and E2), and two TEM-93-producing *E. coli* isolates from Kraków (fingerprint G). The older TEM-48-producing *K. pneumoniae* isolate from Rzeszów and the TEM-29-producing *E. coli* isolate from Suwałki possessed plasmids with unique restriction patterns.

Antimicrobial susceptibility testing of the clinical isolates and their transconjugants. The clinical isolates and their transconjugants were subjected to susceptibility testing (Table 3). All the organisms analyzed demonstrated MIC patterns that are typical for ESBL producers, with significantly elevated MICs of the majority of β-lactams tested and the remarkable effect of inhibitors on the MICs of selected compounds. TEM-29-producing isolate *E. coli* SU2947/98 in general exhibited low-level resistance to β-lactam antibiotics. TEM-85 producers were characterized by relatively low MICs of cephalothin (MICs, 16 to 64 μg/ml) compared to those of ceftazidime (MICs, 64 to >512 μg/ml). In the majority of isolates the MICs of ceftazidime were clearly higher than the cefotaxime MICs (TEM-29, TEM-47, TEM-85, TEM-86, and TEM-93 producers), and it was especially profound in the case of TEM-85-producing isolates (cefotaxime MICs, 1 to 4 μg/ml). The opposite situation was observed in TEM-4-producing *K. pneumoniae* CZ9455/99 and CZ9459/99 and *K. oxytoca* BB1753/00; however, apart from TEM-4, these isolates also expressed the cefotaxime-hydrolyzing CTX-M-3 enzyme (4). Only TEM-94 producers showed fully comparable levels of resistance to ceftazidime and cefotaxime.

In general, the MICs of the transconjugants reflected well the patterns observed in clinical isolates. Transconjugants of isolates *K. pneumoniae* CZ9455/99 and CZ9459/99 and *K. oxytoca* BB1753/00 expressed only TEM-4 and, therefore, were characterized by comparable MICs of cefotaxime and ceftazidime (MICs, 4 to 8 μg/ml).

Cloning of bla_{TEM} genes and characterization of the *E. coli* transformants. In order to characterize the new TEM variants, the bla_{TEM-85}, bla_{TEM-86}, bla_{TEM-93}, and bla_{TEM-94} genes were cloned together with their promoters and expressed in the isogenic *E. coli* background. The only nucleotide differences between the DNA fragments cloned were those located in the coding regions of the genes. The resulting constructs were designated pGBT-85, pGBT-86, pGBT-93, and pGBT-94, respectively. The susceptibility profiles of the transformants were characterized by evaluation of the MICs (Table 3). The TEM-85-producing strain was characterized by much higher MICs of ceftazidime and aztreonam (MICs, 256 and 128 μg/ml, respectively) than those of cefotaxime and cephalothin (MICs, 2 and 16 μg/ml, respectively). In the case of the TEM-86-producing transformant, the MICs of ceftazidime and aztreonam were significantly diminished (MICs, 64 and 8 μg/ml, respectively) and the cephalothin MIC was much increased (MIC, 256 μg/ml) compared to those for the TEM-85 producer. The resistance levels of the TEM-93- and TEM-94-producing strains were very similar to each other, and they demonstrated almost even MICs of cefotaxime, ceftazidime, and aztreonam (MICs, 2 to 8 μg/ml).

DISCUSSION

The analysis of 17 *K. pneumoniae*, *K. oxytoca*, and *E. coli* isolates collected from five hospitals in Poland from 1998 to 2000 has much enriched the view of the possible evolution of TEM ESBLs in Poland, as proposed in our previous studies (15, 19). They expressed seven TEM ESBL variants, of which only TEM-47 had been observed before (15, 18, 19). The other variants were TEM-4 and TEM-29, which were originally described in France (2, 34), and four novel enzymes, TEM-85, TEM-86, TEM-93, and TEM-94. The bla_{TEM-4} gene was identical to that identified in France (34), which suggested that it could have appeared in Poland due to importation, although one also cannot exclude the possibility that it emerged independently. On the other hand, the bla_{TEM-1F}-specific silent mutations clearly differentiated the bla_{TEM-29} gene reported here from its bla_{TEM-1B}-derived "French" counterpart (2). This indicated that bla_{TEM-29} emerged in Poland due to convergent evolution.

The detailed comparative analysis of the coding regions of all the bla_{TEM} genes identified in Poland revealed a relatively compact, putative view of their evolutionary tree (Fig. 1). It could consist of two major parts that originated from bla_{TEM-1A} and bla_{TEM-1F} precursor genes, respectively. The bla_{TEM-1A} branch has led to the bla_{TEM-93} gene, and it could have proceeded through the stepwise acquisition of three nonsynonymous mutations, with the intermediary variants not yet

TABLE 3. Antimicrobial susceptibilities of the clinical isolates; their *E. coli* A15 transconjugants; and the TEM-85-, TEM-86-, TEM-93-, and TEM-94-producing *E. coli* DH5α transformants

Strain no. and ESBL variant	MIC (μg/ml)[a]													
	AMP	PIP	TZP	CEF	CTX	CTX + CLA	CAZ	CAZ + CLA	ATM	ATM + CLA	FOX	IPM	AMK	GEN
K. pneumoniae SU2949/98 TEM-85	>512	>512	128	64	2	0.5	512	2	128	0.5	32	0.125	256	64
K. pneumoniae SU3220/98 TEM-85	>512	>512	4	32	1	0.06	512	2	256	0.25	4	0.125	256	128
K. pneumoniae SU3519/98 TEM-85	>512	>512	8	64	4	0.06	>512	4	512	1	4	0.125	256	256
E. coli SU3408/98 TEM-85	>512	256	≤0.5	16	1	≤0.03	64	0.25	32	0.06	4	0.25	128	128
K. pneumoniae SU2946/98 TEM-86	>512	128	4	256	4	0.06	128	1	16	0.125	4	0.125	256	64
E. coli SU2947/98 TEM-29	256	256	16	32	0.25	0.06	4	0.125	0.25	0.06	2	0.25	2	1
K. pneumoniae GD3658/98 TEM-47	>512	>512	32	>512	8	0.25	128	1	128	0.125	8	0.125	1	32
K. pneumoniae GD3661/98 TEM-47	>512	>512	4	>512	32	0.25	256	1	512	0.25	32	0.125	1	32
K. pneumoniae GD3670/98 TEM-47	>512	>512	16	>512	16	0.25	128	1	128	0.125	32	0.125	1	64
E. coli GD3667/98 TEM-47	>512	256	≤0.5	256	8	≤0.03	16	0.125	16	≤0.03	2	0.125	2	128
E. coli GD3657/98 TEM-94	>512	256	1	>512	32	0.06	32	0.25	16	0.06	8	0.125	64	256
E. coli GD3668/98 TEM-94	>512	>512	2	>512	128	1	64	1	64	0.5	32	1	16	32
E. coli KR 3445/98 TEM-93	>512	256	2	512	4	0.06	64	0.25	64	0.06	8	0.125	1	0.5
E. coli KR 3447/98 TEM-93	>512	512	2	512	8	0.06	64	0.5	64	0.06	8	0.125	1	0.5
K. pneumoniae CZ 9455/99 TEM-4 (+CTX-M-3)[b]	>512	>512	32	>512	256	2	64	2	64	0.5	128	0.125	>256	>256
K. pneumoniae CZ 9459/99 TEM-4 (+CTX-M-3)[b]	>512	>512	64	>512	256	2	64	2	64	0.5	64	0.25	>256	>256
K. oxytoca BB 1753/98	>512	512	1	>512	64	0.06	4	0.125	4	0.125	64	0.25	>256	>256
TEM-4 (+CTX-M-3)														
R^+ (*E. coli* SU3408/98) TEM-85	>512	128	≤0.5	8	0.25	≤0.03	64	0.25	16	0.06	4	0.5	1	16
R^+ (*K. pneumoniae* GD3661/98, GD3670/98, and GD3667/98) TEM-47	>512	64–128	≤0.5	128	1–2	≤0.03	16–32	0.125	8–16	≤0.03	1–2	0.125	0.5–1	16–32
R^+ (*E. coli* GD3657/98 and GD3668/98) TEM-94	>512	128	≤0.5	128	4	≤0.03	8–16	0.125	2	≤0.03	1	0.125	4	32
R^+ (*E. coli* KR3445/98) TEM-93	>512	256	≤0.5	256	2	≤0.03	32	0.125	16	≤0.03	2	0.25	0.5	0.5
R^+ (*K. pneumoniae* CZ9455/99 and CZ9459/99) TEM-4	>512	64–128	1–2	64–128	4–8	≤0.03	4	0.06	2–4	≤0.03	2	0.125	16–32	64
R^+ (*K. oxytoca* BB1753/98) TEM-4	>512	64	1	128	4	≤0.03	4	0.125	2	≤0.03	2	0.125	>256	>256
E. coli DH5α(pGBT-85) TEM-85	>512	128	≤0.5	16	2	≤0.03	256	0.25	128	0.25	2	0.25	ND	ND
E. coli DH5α(pGBT-86) TEM-86	>512	32	≤0.5	256	4	≤0.03	64	0.25	8	0.125	2	0.25	ND	ND
E. coli DH5α(pGBT-93) TEM-93	>512	128	≤0.5	128	2	≤0.03	8	0.06	8	≤0.03	2	0.125	ND	ND
E. coli DH5α(pGBT-94) TEM-94	>512	128	≤0.5	128	4	≤0.03	8	0.06	4	≤0.03	2	0.125	ND	ND
E. coli A15 R^-	2	≤0.5	≤0.5	2	≤0.03	≤0.03	0.125	0.125	0.06	≤0.03	1	0.125	0.5	0.25
E. coli DH5α	2	≤0.5	≤0.5	2	≤0.03	≤0.03	0.125	0.06	0.06	≤0.03	2	0.125	ND	ND
E. coli ATCC 25922	4	2	2	8	0.06	≤0.03	0.25	0.125	0.06	0.06	4	0.125	1	0.5

[a] Abbreviations: AMP, ampicillin; PIP, piperacillin; TZP, piperacillin with tazobactam; CEF, cephalothin; CTX, cefotaxime; CLA, clavulanate; CAZ, ceftazidime; ATM, aztreonam; FOX, cefoxitin; IPM, imipenem; AMK, amikacin; GEN, gentamicin; ND, not determined; R^+, transconjugants; R^-, *E. coli* strain without plasmids, used as a recipient in mating.

[b] Some of the MICs for these isolates were published previously (4).

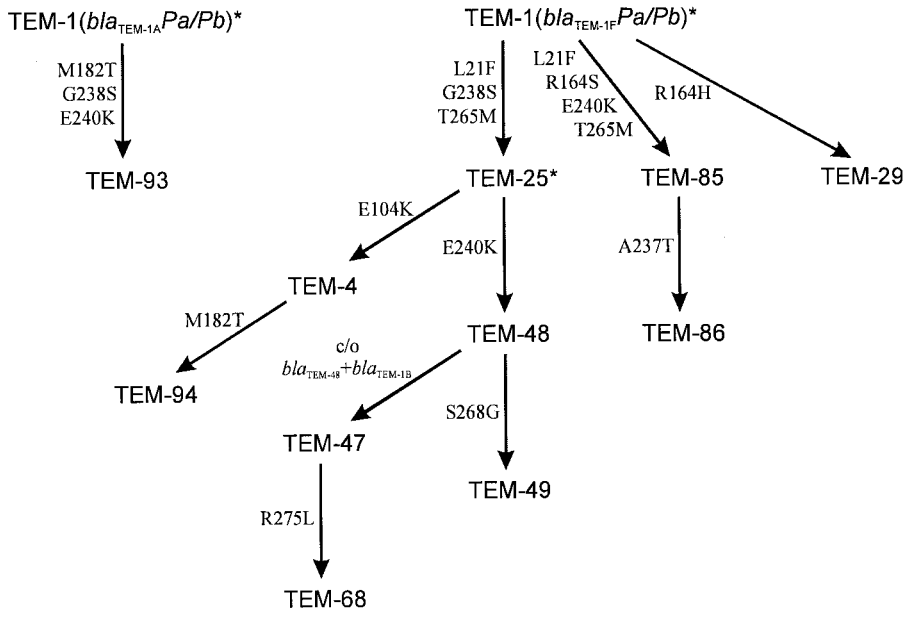

FIG. 1. Possible view of the evolution of TEM ESBL variants observed in Poland so far. A part of the scheme containing the TEM-47, TEM-48, TEM-49, and TEM-68 variants has been proposed earlier (15, 19). Asterisks indicate the enzymes (TEM-24) or the enzymes encoded by genes (TEM-1 encoded by $bla_{\text{TEM-1A}}$ [*Pa*/*Pb*] and $bla_{\text{TEM-1F}}$ [*Pa*/*Pb*]) that have not yet been identified in Poland or not at all, respectively. c/o $bla_{\text{TEM-48}}$ + $bla_{\text{TEM-1B}}$, the hypothetical crossing-over event that could give rise to the $bla_{\text{TEM-47}}$ sequence (19).

identified in Poland. The nine $bla_{\text{TEM-1F}}$-related variants have most probably evolved along three separate branches. One of these has included only $bla_{\text{TEM-29}}$, which differs from $bla_{\text{TEM-1F}}$ by a single mutation. The second lineage has contained two genes, $bla_{\text{TEM-85}}$ and $bla_{\text{TEM-86}}$, of which $bla_{\text{TEM-86}}$ most likely emerged from $bla_{\text{TEM-85}}$ by acquisition of a single mutation. $bla_{\text{TEM-85}}$ differs from $bla_{\text{TEM-1F}}$ by four mutations, and none of the intermediates has been observed in Poland to date. The structure of the third hypothetical branch has been the most complex, and its view has developed gradually with time (15, 19). It may consist of the genes described earlier, $bla_{\text{TEM-47}}$, $bla_{\text{TEM-48}}$, $bla_{\text{TEM-49}}$, and $bla_{\text{TEM-68}}$, and the $bla_{\text{TEM-4}}$ and $bla_{\text{TEM-94}}$ genes identified in this work. The $bla_{\text{TEM-94}}$ gene is a single-point mutant of $bla_{\text{TEM-4}}$, which suggests direct evolution. On the other hand, $bla_{\text{TEM-4}}$ differs from $bla_{\text{TEM-25}}$ by only one mutation (10), which indicates that the latter gene could have been a possible precursor of $bla_{\text{TEM-4}}$. As mentioned above, $bla_{\text{TEM-25}}$ has not been identified in Poland to date; but it was proposed to be the ancestor of the $bla_{\text{TEM-47}}$, $bla_{\text{TEM-48}}$, $bla_{\text{TEM-49}}$, and $bla_{\text{TEM-68}}$ genes (15, 19). Therefore, it is possible that $bla_{\text{TEM-25}}$ gave rise to two separate subbranches in the evolution of the $bla_{\text{TEM-1F}}$-derived ESBL genes observed in Poland, which could have proceeded in part in France.

Analysis of the sequences located upstream from the bla_{TEM}-coding regions has confirmed in general the evolutionary hypotheses drawn from the comparison of the coding parts. Almost all the genes were driven by the strong overlapping promoter *Pa*/*Pb* (26, 27), which suggested that the $bla_{\text{TEM-1A}}$ and $bla_{\text{TEM-1F}}$ alleles, which could have been the precursors of "Polish" bla_{TEM} genes, were driven by *Pa*/*Pb* rather than by *P3* and *P4*, respectively, as reported to date (27). The only exception was $bla_{\text{TEM-49}}$, which was found to have the *P3* promoter (26, 27). This indicated that the particular allele of this gene identified in one of our previous studies had not been a direct, one-step descendant of $bla_{\text{TEM-48}}$ from the same study (19) and, for example, could have acquired its promoter due to homologous recombination.

The scheme for the evolution of the bla_{TEM} gene indicates the existence of genetic links between bacterial isolates producing different but closely related TEM variants in terms of the similarities of their PFGE patterns and/or plasmid fingerprints. However, the only clear examples of such links were observed within a single hospital, for example, between TEM-47- and TEM-68-producing *K. pneumoniae* isolates in Wrocław (15) and between *K. pneumoniae* with TEM-85 and TEM-86 in Suwałki. On the other hand, significant genetic diversity was observed among the isolates from different centers, exemplified mostly by the diversity of the plasmids carrying the $bla_{\text{TEM-48}}$ and $bla_{\text{TEM-47}}$ genes or the $bla_{\text{TEM-4}}$ and $bla_{\text{TEM-94}}$ genes. These data might indicate an important contribution of convergent evolution; however, bla_{TEM} genes could have also been transposed between different plasmids and/or the plasmids could have diversified due to multiple recombination events. The center-to-center transmission of TEM ESBL-producing strains was documented only by similarities between the isolates that expressed the same enzyme variant. For example, the clonal relatedness of TEM-47-producing *K. pneumoniae* from Łódź, Warsaw, and Wrocław was demonstrated in our earlier work (15, 18); and this study showed the similarities of their $bla_{\text{TEM-47}}$-carrying plasmids to those present in isolates from Gdańsk. Considering the dynamics of bacterial evolution under the pressure of antibiotic use, one must realize that a study of a relatively small group of isolates collected over a longer period in several hospitals usually results in only a fragmentary view of their epidemiology.

Many of the substitutions identified in the TEM enzymes analyzed here were those that significantly affect β-lactamase activity and that confer a selective advantage to bacterial strains. First, these were the mutations responsible for ESBL activity, R164H, R164S, and G238S (25). Second, the E104K or E240K substitution, which enhances enzyme interactions with ceftazidime and aztreonam (25), was observed in all the variants except TEM-29. Third, the intragenic suppressor mutation M182T (23) was found in TEM-93 and TEM-94. Fourth, the R275L substitution, identified in TEM-68, diminishes the effects of β-lactamase inhibitors (15, 22). Finally, the A237T mutation was observed in TEM-86, and TEM-85 and TEM-86 have been the third pair of natural TEM β-lactamase variants that differ from each other only by this mutation. In the comparative analysis of TEM-46 and TEM-24, the stimulatory effect of A237T on their catalytic efficiencies against cephems (cephalothin and cefotaxime) and negative effect on catalytic efficiencies against penams were observed (12). In the comparison of TEM-10 and TEM-5, the clear increases in the cephalothin and cefotaxime MICs were accompanied by decreases in the MICs of amoxicillin, ceftazidime, and aztreonam; therefore, this mutation was described as modulating the activities of the enzymes against various substrates (7). In the analysis of TEM-85 and TEM-86, the A237T mutation exerted a strong positive effect on the cephalothin MIC, while decreases in resistance to piperacillin, ceftazidime, and aztreonam were observed. No significant differences in the cefotaxime MIC were found; however, the cefotaxime MIC for the TEM-85 producer was already high compared to those for the TEM-46 and TEM-10 producers (7, 12). Our data confirm the hypothesis of the modulating effect of the A237T mutation (7).

In the 1998 survey of ESBL types among the enterobacteria in Polish hospitals, β-lactamases of the TEM family were produced by 20.1% of the isolates collected over a 4-month period. Their frequency was similar to that of the CTX-M β-lactamases (18.8%) but far below that of the SHV-type enzymes (60.4%) (Gniadkowski et al., unpublished). The data presented in this report and in our earlier reports (3, 5, 15, 19, 20) clearly show that although TEM β-lactamases are not the most prevalent, they have certainly been the most diversified ESBLs. Using large amounts of expanded-spectrum cephalosporins and often lacking proper infection control measures, Polish hospitals have created good selective conditions for the rapid evolution of β-lactamase-mediated resistance.

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