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Development of "Oligotyping" for Characterization and Molecular Epidemiology of TEM β-Lactamases in Members of the Family *Enterobacteriaceae*

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Based on the DNA sequences of bla_{TEM-1} and bla_{TEM-2}, which encode parental penicillinases TEM-1 and TEM-2, respectively, and of bla_{TEM-3}, bla_{TEM-4}, bla_{TEM-5}, bla_{TEM-6}, and bla_{TEM-7}, which encode extended-spectrum β-lactamases, we designed heptadecanucleotides to discriminate point mutations in five loci. Determination of the hybridization profiles by colony hybridization with this selection of probes, termed "oligotyping," allowed characterization of the TEM variants present in 265 clinical isolates of the family *Enterobacteriaceae* that exhibit synergism between a penicillinase inhibitor and broad-spectrum cephalosporins. Among the 222 strains harboring TEM enzymes, *Klebsiella pneumoniae* (48%) and *Escherichia coli* (21%) were predominant, and TEM-3 was the most common enzyme (60%). Penicillinases TEM-1 and TEM-2 were detected alone (15 and 1%, respectively), combined (1%), or associated with another TEM β-lactamase (17 and 6%, respectively). Fourteen variants, including seven new enzymes, were detected. One, TEM-13, was a new penicillinase with the same isoelectric point and substrate range as TEM-2 but differed by a single amino acid substitution, whereas the others, TEM-14 to TEM-19, were extended-spectrum β-lactamases that consisted of novel combinations of known amino acid substitutions. Different TEM variants were found to coexist within the same cells. A patient could harbor two or three different strains that encoded the same enzyme or two indistinguishable isolates that produced distinct TEM β-lactamases.

β-Lactams constitute one of the most important families of antibiotics, but resistance to these drugs has emerged following their wide use in therapy. Gram-negative bacteria are most often resistant as a result of their production of β-lactamases (17), and numerous enzymes that differ in their substrate ranges have been described (34). The utilization of β-lactams resistant to hydrolysis by penicillinases, such as broad-spectrum cephalosporins, led to the selection of new enzymes. In 1983, 5 years after the introduction of this class of drugs in Europe, Knothe et al. (13) reported transferable resistance to cefotaxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Biochemical and DNA-DNA hybridization studies (12) indicated that the enzyme responsible for this new resistance phenotype was closely related to SHV-1 penicillinase and was designated SHV-2. Starting in 1984, nosocomial outbreaks of multiresistant members of the family *Enterobacteriaceae* highly resistant to cefotaxime and ceftazidime occurred in French hospitals (4, 11, 27, 28). Resistance was due to a new, plasmid-mediated, extended-spectrum β-lactamase named CTX-1 (27). Molecular analysis (30) and nucleotide sequence determination (29) of bla_{TEM-3}, the structural gene for the enzyme, indicated that the β-lactamase was a double point mutation of TEM-2 penicillinase and was therefore redesignated TEM-3 (30). Since then, similar enzymes of either the SHV type (5, 9) or the TEM type (3, 8, 22, 24) have been detected among members of the family *Enterobacteriaceae*. The epidemic aspect of this resistance, the fact that it involves recent cephalosporins and monobactams, and the mechanism of substrate range expansion by point mutations in well-known penicillinases prompted us to study the distribution of TEM β-lactamases in strains of enterobacteria and to develop techniques for the rapid detection and characterization of new variants.

Detection of extended-spectrum TEM enzymes in clinical isolates is conveniently achieved by testing synergy between β-lactamase inhibitors, such as clavulanic acid, and broad-spectrum cephalosporins by the disk-agar diffusion method. However, detailed characterization of the variant enzymes is difficult. Isoelectric focusing is inadequate since the same pI value can correspond to different TEM β-lactamases (Table 1). DNA hybridization with a specific bla_{TEM-1} probe is inappropriate because of cross-hybridization between bla_{TEM} mutants and also because the same isolate can harbor more than one TEM enzyme (22); the same restriction applies to immunological reactions with anti-TEM-1 and -TEM-2 sera (21). Therefore for the discrimination of bla_{TEM} variants, we developed "oligotyping," which consists of the detection of point mutations with oligonucleotide probes. This method, which was first used for TEM-1 and TEM-2 (19), was performed on a collection of 265 clinical isolates of enterobacteria exhibiting synergism between clavulanic acid and a broad-spectrum cephalosporin. We were able to characterize 14 bla_{TEM} genes that encode TEM β-lactamases, including seven new enzymes.

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TABLE 1. TEM extended-spectrum β-lactamases

Enzyme	Gene	pI	Original host	Reference
TEM-3 (CTX-1)	bla_{TEM-3}	6.3	*Klebsiella pneumoniae*	Sirot et al. (27)
TEM-4	bla_{TEM-4}	5.9	*Escherichia coli*	Paul et al. (22)
TEM-5 (CAZ-1)	bla_{TEM-5}	5.55	*Klebsiella pneumoniae*	Petit et al. (24)
TEM-6	bla_{TEM-6}	5.9	*Escherichia coli*	Bauernfeind and Hörl (3)
TEM-7	bla_{TEM-7}	5.41	*Citrobacter freundii*	Gutmann et al. (8)
TEM-8	bla_{TEM-8}	5.9	*Klebsiella pneumoniae*	Mabilat et al.[a]
TEM-9 (RHH-1)	bla_{TEM-9}	5.5	*Klebsiella pneumoniae*	Spencer et al. (32)
TEM-10	bla_{TEM-10}	5.57	*Klebsiella pneumoniae*	Quinn et al. (25)
TEM-11 (CAZ-lo)	bla_{TEM-11}	5.7	*Klebsiella pneumoniae*	Vuye et al. (35)
TEM-12 (TEM-101)	bla_{TEM-12}	5.25	*Escherichia coli*	Gutmann et al. (8), Weber et al.[b]

[a] C. Mabilat, P. Legrand, J. Duval, and P. Courvalin, Program Abstr. 12th Réunion Interdisc. Chimiothér. Antiinfect., abstr. no. 136, 1989.
[a] Enzymes identical to the in vitro mutant TEM-101 were found independently by Weber et al. (D. A. Weber, C. C. Sanders, and J. P. Quinn, 29th ICAAC, abstr. no. 761, 1989) and by us and were named TEM-12.

Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 667, 1989].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The control strains used in this study were *Escherichia coli* K-12 derivatives harboring the following plasmids: pUC18 (bla_{TEM-1}), R6K (bla_{TEM-1} Sm^r), RP4 (bla_{TEM-2} Km^r Tc^r), pCFF04 (bla_{TEM-3} Ak^r Cm^r Km^r Su^r Tc^r) (27), pUD16 (bla_{TEM-4} Km^r Su^r Tc^r Tp^r) (22), pCFF14 (bla_{TEM-5} Km^r Sm^r Su^r Tc^r) (24), pIF100 (bla_{TEM-7} Ak^r Gm^r Su^r Tc^r) (7), p453 (bla_{SHV-1} Cm^r Sm^r Su^r Tc^r) (14), and pLQ26 (bla_{OXA-1} Sm^r Sp^r Su^r) (18). Clinical isolates or transconjugants harboring bla_{TEM-8}, bla_{TEM-9}, bla_{TEM-11}, or bla_{TEM-12} were also included (Table 1). A total of 265 clinical isolates of the family *Enterobacteriaceae*, which were collected from 28 hospitals in five countries from 1984 to 1988, were selected on the basis of synergy between clavulanic acid and a broad-spectrum cephalosporin (cefotaxime, ceftazidime, or ceftriaxone) or of an unusual resistance pattern toward β-lactams. The strains originated in Paris (182 isolates), other cities in France (61 isolates), Belgium (8 isolates), Great Britain (7 isolates), Chile (2 isolates), and the Federal Republic of Germany (1 isolate); 4 isolates were of bovine origin (France). Identification, which was done with the API 20E system (API-bioMérieux, La Balme les Grottes, France), was as follows: *Klebsiella pneumoniae* (159 isolates), *E. coli* (54 isolates), *Enterobacter cloacae* (17 isolates), *Klebsiella oxytoca* (8 isolates), *Serratia marcescens* (7 isolates), *Citrobacter freundii* (3 isolates), *Enterobacter aerogenes* (3 isolates), nontypeable *Salmonella* spp. (3 isolates), "*Salmonella enterica*" serotype Kedougou (2 isolates), *Salmonella typhimurium* (2 isolates), *Proteus morganii* (2 isolates), *K. ozaenae* (2 isolates), *Enterobacter hafniae* (1 isolate), *Enterobacter agglomerans* (1 isolate), and *Citrobacter diversus* (1 isolate). The antibiotic susceptibility patterns were determined by the disk-agar diffusion method (Diagnostics Pasteur, Marnes-la-Coquette, France).

Media. Brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 100 μg of ampicillin per ml was used. Susceptibility tests were done on Mueller-Hinton agar (Diagnostics Pasteur, Marnes-la-Coquette, France). All incubations were done at 37°C.

Preparation of DNA and agarose gel electrophoresis. Purification of plasmid DNA by ultracentrifugation in cesium chloride-ethidium bromide was done as described previously (16). The oligonucleotides used as probes or as primers for the polymerase chain reaction were synthesized by the methoxy phosphoramidite method by using an Applied Biosystems 380B DNA apparatus (Foster City, Calif.). Probes were purified either by 7 M urea, 20% polyacrylamide gel electrophoresis or by high-pressure liquid chromatography on an A-203 (C8) 12.0-nm (4.5 by 250 mm) column by reversed-phase chromatography by using a triethylacetate-ammonium (100 mM)–acetonitrile gradient ranging from 10 to 30%. Amplification primers were used without purification. A 516-bp DNA fragment, internal to bla_{TEM-1}, was amplified by the polymerase chain reaction (26) as described previously (15) by using primers C and H (Table 2). It

TABLE 2. Sequence of the oligonucleotides used for hybridization or amplification[a]

Probe	Sequence	Position
Gln-37	5′-d[ACCCAACTGATCTTCAG]-3′ Gln	325
Lys-37	5′-d[ACCCAACTTATCTTCAG]-3′ Lys	325
Glu-102	5′-d[TGAGTACTCAACCAAGT]-3′ Glu	520
Lys-102	5′-d[TGAGTACTTAACCAAGT]-3′ Lys	520
Arg-162	5′-d[TTCCCAACGATCAAGGC]-3′ Arg	700
Ser-162	5′-d[TTCCCAACTATCAAGGC]-3′ Ser	700
His-162	5′-d[GTTCCCAATGATCAAGG]-3′ His	701
Gly-236	5′-d[ACGCTCACCGGCTCCAG]-3′ Gly	922
Ser-236	5′-d[ACGCTCACTGGCTCCAG]-3′ Ser	922
Thr-Lys	5′-d[ACGCTTACCGGTTCCAG]-3′ Lys Thr	922
Thr 261	5′-d[TCCCCGTCGTGTAGATA]-3′ Thr	998
Met 261	5′-d[TCCCCGTCATGTAGATA]-3′ Met	998
Amplification primers		
C	5′-d[CCCCGAAGAACGTTTTC]-3′	385
H	5′-d[ATCAGCAATAAACCAGC]-3′	901

[a] The number in the position column corresponds to the position of the first 5′ base of the oligonucleotide by the numbering of Sutcliffe (33); primer C is identical to the leading strand; primer H and all probes are identical to the lagging strand. Designation of the probes refers to the substituted amino acid in the TEM enzyme; numbers indicate the position of the amino acid in the premature protein according to Sutcliffe (33). The 17-mers are centered on positions where mutations (underlined) lead to the amino acid substitutions indicated under the sequence (Table 3).

encompasses the *Hin*cII-*Bgl*I fragment that is generally used as a probe for bla_{TEM} genes. The amplified DNA was electrophoresed in 0.8%, low-temperature-gelling agarose type VII (Sigma, St. Louis, Mo.) and purified as described previously (16).

DNA-DNA hybridization. Plasmid pUC18 DNA and the polymerase chain reaction-generated bla_{TEM-1} internal fragment were labeled with [α-^{32}P]dCTP (3000 Ci/mmol) by nick translation (16). Oligonucleotide probes (5 pmol) were ^{32}P end-labeled by using T4 polynucleotide kinase and 5′-[γ-^{32}P] ATP (5,000 Ci/mmol) for 30 min. Colony hybridization was performed as follows. Nylon filters (7.5 by 11.5 cm) (NY N13; Schleicher and Schüell Co., Dassel, Federal Republic of Germany) were placed on Mueller-Hinton agar in MA 1701 R plates (Dynatech Co., Chantilly, Va.) containing ampicillin (100 μg/ml) and inoculated with 9-h broth cultures by using a 96-tip M 391 manual inoculator (Dynatech Co.). After 14 h of incubation, colonies were lysed and DNA was fixed on filters as described previously (16). Hybridization with plasmid and fragment probe DNA was done under stringent conditions in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–0.7% nonfat dry milk at 65°C overnight. Washings were done twice for 30 min each time at room temperature in 2× SSC–0.25% nonfat dry milk–0.1% sodium dodecyl sulfate buffer and twice for 1 h each time at 70°C in 0.1× SSC–0.1% sodium dodecyl sulfate (10). Hybridization with oligonucleotides under relaxed conditions was performed simultaneously on duplicate filters for 3 h at 37°C in 6× SSC–0.7% nonfat dry milk. Washings were done under conditions that differentiated point mutations (37): twice for 5 min each time and then twice for 30 min each time at 4°C in 5× SSC, 15 min at 37°C in 3 M tetramethylammonium chloride–50 mM Tris hydrochloride (pH 8.0)–2 mM EDTA–1 mg of sodium dodecyl sulfate per ml, and a minimum of 10 min at 51 to 52°C in the same solution until positive and negative control dots were as expected by using a Geiger-Muller counter. Autoradiography on RX films (Fuji Co., Tokyo, Japan) was performed at −20°C overnight or longer if necessary. Region 236 appeared to be difficult to study. If the Thr-235–Lys-237 probe, which was intended to detect two point mutations in the 17-bp region, gave the expected results, the Gly-236 and Ser-236 probes tended to cross-hybridize, probably because of their high G+C content (70 and 65%, respectively) or because of homology with an unknown DNA region. This problem was solved by washing the filters in 6× SSC, three times for 5 min each time at 4°C, once for 15 min at room temperature, and several times for 10 min each time at 60°C.

Isoelectric focusing. Analytical isoelectric focusing was performed in precast polyacrylamide gels (pH 5.0 to 8.0) by using a Pharmacia (Uppsala, Sweden) PhastSystem apparatus according to the instructions of the manufacturer. Enzymes TEM-1, TEM-2, TEM-3, and TEM-4 were used as standards for pI determinations. β-Lactamase activity was revealed with a 0.5-mg/ml solution of nitrocefin (Glaxo, Greenford, United Kingdom).

Enzymes and chemicals. T4 polynucleotide kinase was purchased from Amersham International (Amersham, England), lysozyme was purchased from Sigma, and *Taq* polymerase was purchased from Perkin Elmer-Cetus (Norwalk, Conn.). The nick translation kit was from Bethesda Research Laboratories. Radionucleotides were from Amersham International. Tetramethylammonium chloride was from Aldrich (St. Louis, Mo.).

RESULTS

Design of oligonucleotides and oligotyping. Plasmid-mediated penicillinases TEM-1 and TEM-2 can be discriminated by their pIs, 5.4 and 5.6, respectively, a difference which results from an amino acid substitution at position 37 of the premature protein (according to the numbering of Sutcliffe [33]); TEM-1 has a glutamine, whereas TEM-2 has a lysine, following a transversion from a cytosine to an adenine at nucleotide position 317 (6). In clinical isolates, detection of each alternative base at this locus can be achieved by colony hybridization by using two oligonucleotide probes complementary to either sequence (20). The sequences of structural genes bla_{TEM-3}, bla_{TEM-4}, bla_{TEM-5}, bla_{TEM-6}, and bla_{TEM-7} for TEM extended-spectrum β-lactamases have been determined previously (7, 29, 31; S. Goussard, W. Sougakoff, C. Mabilat, A. Bauernfeind, and P. Courvalin, unpublished data). The genes possess point mutations which either are silent or can result in amino acid substitutions. Certain substitutions have no consequence on the enzymatic activity, whereas others, such as lysine at position 102, serine at position 162, serine at position 236, and the doublet threonine-lysine at positions 235 and 237, respectively, extend the substrate range (7, 29, 31). Positions differentiating TEM-1, TEM-2, TEM-3, TEM-4, TEM-5, TEM-6, and TEM-7 were selected for this study (Table 3), and the corresponding oligonucleotides were synthesized (Table 2). The probes were 17 bases long and centered on the mismatch, a feature which generates the greatest destabilizing effect (36). Lack of homology of the oligonucleotides with other genomic regions of members of the family *Enterobacteriaceae* was tested by comparison with the nucleotide sequence data of GenBank. Study of a gene for a point mutant enzyme by using the selection of probes (Table 2) yielded a hybridization profile that we termed oligotype. For example, the oligotype of bla_{TEM-1} was Gln-37$^+$, Lys-37$^-$, Glu-102$^+$, Lys-102$^-$, Arg-162$^+$, His-162$^-$, Ser-162$^-$, Gly-236$^+$, Ser-236$^-$, Thr-235$^-$ Lys-237$^-$, Thr-261$^+$, and Met-261$^-$. Combination of the oligotypes of the genes for two TEM-derived enzymes in the same isolate resulted in a profile which allowed us to detect them. Characterization of three coresident enzymes is possible except if two of the TEM variants differ at a single position, e.g., TEM-1 and TEM-2, TEM-3 and TEM-14, TEM-3 and TEM-15, TEM-4 and TEM-15, TEM-6 and TEM-16, TEM-7 and TEM-12, and TEM-17 and TEM-18. Thus, TEM-1+TEM-2+TEM-3 is interpreted as TEM-1+TEM-3 (Table 3). This technical limitation can lead to underestimation of the prevalence of certain enzymes. A new variant is detected when the oligotype obtained differs from the expected mutation combinations. The following two possibilities exist: (i) the mutation corresponds to a new combination of known mutations, e.g., TEM-7 has a lysine at position 37 like TEM-2 and a serine at position 162 like TEM-5, or (ii) the mutation, because of an as yet undetected substitution, has no homology with known alternatives at a given locus. This mutation may be responsible for an amino acid change or may be silent. Variants escaped detection if mutations occurred in nonprobed loci.

The oligotyping method consists of a series of hybridizations with three types of probes: (i) plasmid pUC18 which contains, in addition to bla_{TEM-1}, a DNA fragment encoding the first 146 amino acids of the β-galactosidase that is present in the majority of the *Enterobacteriaceae*, to test for the presence of target DNA on the filter, (ii) the bla_{TEM-1} internal probe to detect the presence of a bla_{TEM} gene(s),

TABLE 3. Amino acid substitutions in TEM β-lactamases[a]

Enzyme	Amino acid at position[b]:					Reference or source
	37	102	162	236	261	
Penicillinases						
TEM-1	Gln	Glu	Arg	Gly	Thr	33
TEM-2	Lys					1
TEM-13	Lys				Met	This study
β-Lactamases with one substitution enlarging the catalytic activity						
TEM-17		*Lys*				This study
TEM-18	Lys	*Lys*				This study
TEM-6		*Lys*	His[c]			This study
TEM-16	Lys	*Lys*	His			This study
TEM-7	Lys		*Ser*			7
TEM-12 (TEM-101)			*Ser*			This study
TEM-19				*Ser*		This study
β-Lactamases with two substitutions enlarging the catalytic activity						
TEM-9		*Lys*	*Ser*		Met	This study
TEM-3	Lys	*Lys*		*Ser*		30
TEM-4		*Lys*		*Ser*	Met	31
TEM-14	Lys	*Lys*		*Ser*	Met	This study
TEM-15		*Lys*		*Ser*		This study
TEM-5			*Ser*	*Thr-Lys*[d]		31
β-Lactamase with three substitutions enlarging the catalytic activity: TEM-8	Lys	*Lys*	*Ser*	*Ser*		This study
β-Lactamase with a new mutation: TEM-11	Lys		His	?		This study

[a] Amino acid sequences were deduced from the nucleotide sequences of bla_{TEM-1} to bla_{TEM-7}, which constituted the data base for the design of oligonucleotides. Genes for TEM-8 (Mabilat et al., Program Abstr. 12th Réunion Interdisc. Chimiothér. Antiinfect., abstr. no. 136, 1989) and TEM-9 (32) were oligotyped in this study.

[b] Amino acid numbering was that of Sutcliffe (33). Residues involved in the extension of the substrate range are indicated in italics.

[c] The enzymatic consequence of the His-162 substitution is not known.

[d] The double mutation responsible for two amino acid substitutions was Ala for Thr at position 235 and Glu for Lys at position 237. This combination was considered as a single entity.

and (iii) the selection of 12 oligonucleotides to characterize the mutant bla_{TEM} derivatives.

Detection of TEM enzymes by oligotyping and comparison with isoelectric focusing. In order to validate the oligotyping method, probes were hybridized to DNA of reference strains harboring TEM, OXA, and SHV enzymes or no β-lactamase (see Materials and Methods). A perfect correlation was found between the predicted and the observed oligotypes of bla_{TEM-1} to bla_{TEM-5} and bla_{TEM-7} (Table 3). No hybridization was observed with strains that encoded OXA-1 and SHV-1 β-lactamases or that were devoid of enzyme. Twenty-four isolates belonging to five species (1 *Enterobacter aerogenes*, 1 *Enterobacter cloacae*, 2 *Escherichia coli*, 19 *Klebsiella pneumoniae*, and 1 *Serratia marcescens*) that have already been tested by isoelectric focusing for the presence of β-lactamases (and containing TEM-2, TEM-3, TEM-2 plus TEM-3, or SHV-2; A. Philippon, unpublished data) were studied by oligotyping. The results obtained by the two techniques were identical, as was found in a previous study that tested the locus that discriminates between TEM-1 and TEM-2 (20).

Distribution of TEM enzymes in the *Enterobacteriaceae*. The oligotypes of the 265 strains collected were determined. Forty-three strains did not produce a TEM enzyme. Among the 222 (considered as 100%) remaining strains (Table 4), penicillinases TEM-1 and TEM-2 were detected alone (15 and 1%, respectively) combined (1%), or associated with an extended-spectrum TEM variant (17 and 6%, respectively). Strains that did not produce a TEM enzyme or that encoded TEM-1, TEM-2, or both must harbor another extended-spectrum enzyme, presumably of the SHV type, since the strains were collected on the basis of synergy between clavulanic acid and broad-spectrum cephalosporins. Detection of the gene for SHV enzymes by hybridization is difficult since nearly all *Klebsiella pneumoniae* encode SHV-1 or its putative chromosomal ancestor LEN-1 (2). Therefore, restriction fragment probes for genes encoding SHV-type enzymes would cross-hybridize.

The remaining 183 (82%) strains produced a TEM enzyme with an extended spectrum that was sometimes associated with a TEM-1 or TEM-2 penicillinase. As already reported (27), the most common species were *Klebsiella pneumoniae* (96 strains, 43%) and *Escherichia coli* (30 strains, 13%). The distributions of enzymes in various bacterial species are indicated in Table 4. TEM-3 was found in 133 (60%) isolates belonging to 13 species isolated in various parts of France but not in other countries. The other TEM enzymes were less common. TEM-4 was associated with TEM-3 in a strain of *Enterobacter cloacae*. TEM-5 and TEM-7 were not detected. TEM-6 and TEM-8 were present in *Escherichia coli* and *Klebsiella pneumoniae* isolates, respectively, originating from different geographical areas than the reference strains. TEM-9 (RHH-1), which was first described in Great Britain (32), was not found. Certain oligotypes could not be assigned to a known enzyme and corresponded to new variants (Table 3). Ten genes were characterized. Three

TABLE 4. Distribution of TEM enzymes in 222 clinical isolates of the family *Enterobacteriaceae* determined by oligotyping

Enzyme[a]	Organism	No. of strains	pI[b]
TEM-1	—[c]	35	
TEM-2	*Serratia marcescens*	2	
	Enterobacter cloacae	1	
TEM-1 + TEM-2	*Enterobacter cloacae*	2	
TEM-3	—[d]	98	
TEM-3 + TEM-1	—[e]	29	
TEM-3 + TEM-2	*Klebsiella pneumoniae*	5	
TEM-4 + TEM-3	*Enterobacter cloacae*	1	
TEM-6[f]	*Escherichia coli*	1	
TEM-6 + TEM-1	*Escherichia coli*	1	
TEM-8	*Klebsiella pneumoniae*	3	
TEM-8 + TEM-1	*Klebsiella pneumoniae*	1	
	Escherichia coli	1	
TEM-9[f]	*Klebsiella pneumoniae*	1	
TEM-11	*Escherichia coli*	1	5.7
TEM-11 + TEM-2	*Klebsiella pneumoniae*	7	
TEM-12 (TEM-101)	*Escherichia coli*	3	5.25
	Klebsiella pneumoniae	1	
TEM-13	*Proteus morganii*	1	5.6
TEM-14 + TEM-1	*Klebsiella pneumoniae*	2	6.3
TEM-15	*Klebsiella pneumoniae*	8	6
	Klebsiella oxytoca	1	
TEM-15 + TEM-1	*Klebsiella pneumoniae*	1	
TEM-15 + TEM-3	*Klebsiella pneumoniae*	1	
TEM-16 + TEM-1	*Klebsiella pneumoniae*	1	6.3
TEM-17	*Klebsiella pneumoniae*	1	5.9
TEM-18	*Klebsiella pneumoniae*	2	6.3
TEM-18 + TEM-2	*Klebsiella pneumoniae*	1	
TEM-19	*Escherichia coli*	1	5.4
	Klebsiella pneumoniae	2	
TEM-19 + TEM-1	*Klebsiella pneumoniae*	1	
Unexplained phenotypes			
A	*Klebsiella pneumoniae*	4	6
B	*Klebsiella pneumoniae*	2	6.8

[a] The strain harboring TEM-10 (25) was not available.

[b] The isoelectric points of the new variants are indicated.

[c] TEM-1 was found in *Citrobacter freundii* (n = 1), *Enterobacter cloacae* (n = 2), *Escherichia coli* (n = 19), *Klebsiella pneumoniae* (n = 10), *Serratia marcescens* (n = 2), and *Salmonella typhimurium* (n = 1).

[d] TEM-3 was found in *Citrobacter freundii* (n = 1), *Enterobacter aerogenes* (n = 3), *Enterobacter cloacae* (n = 7), *Escherichia coli* (n = 15), *Klebsiella oxytoca* (n = 5), *Klebsiella pneumoniae* (n = 29), *Proteus morganii* (n = 1), *Salmonella enterica* serotype Kedougou (n = 1), *Serratia marcescens* (n = 2), nontypeable *Salmonella* (n = 3), and *Salmonella typhimurium* (n = 1).

[e] TEM-1 + TEM-3 were found in *Citrobacter diversus* (n = 1), *Enterobacter agglomerans* (n = 1), *Escherichia coli* (n = 4), and *Klebsiella pneumoniae* (n = 23).

[f] Reference strains (3, 32).

encoded variants, TEM-8, TEM-11 (Caz-lo), and TEM-12 (TEM-101), that have already been described at the phenotype level (8, 32, 35); six encoded new extended-spectrum β-lactamases, designated TEM-14 to TEM-19; and one specified a new penicillinase, TEM-13. The pI of the latter enzyme and the resistance phenotype of the *Proteus morganii* host were indistinguishable from those of a TEM-2-producing strain. It thus appears that substitution of a methionine for a threonine at position 261 does not alter the substrate range nor the pI of the enzyme, an observation that explains why TEM-13 remained undetected. The new TEM-14 to TEM-19 extended-spectrum enzymes were present in 43 (19%) strains. They were all detected in *Klebsiella pneumoniae*, and some of them, e.g., TEM-15 and TEM-19, were also detected in other species of enterobacteria. Certain variants (e.g., TEM-14 and TEM-15 relative to TEM-3 and TEM-4) were identical to known enzymes for residues involved in the extension of the enzymatic substrate range (positions 102; 162; and 235, 236, and 237) but differed at other positions (Table 3).

In six strains that fell into two groups (A, B), there was a discrepancy between the oligotype and the expected resistance phenotype (Table 4). This could result from the existence of as yet nondetected mutations in extended-spectrum variants.

Among the 222 strains that produced TEM β-lactamases, the coexistence of two enzymes such as TEM-1 and TEM-2 (1%) or of an extended-spectrum TEM enzyme with TEM-1 (17%) or TEM-2 (6%) was common. TEM-3 was present alone in 45% of the strains or was associated with TEM-1 (13%) or TEM-2 (2%). Two enzymes with extended spectra could also coexist, e.g., TEM-3 and TEM-4 in an *Enterobacter cloacae* isolate and TEM-3 and TEM-15 in a strain of *Klebsiella pneumoniae*. We did not find the association of three enzymes within the same cell, but as mentioned above, oligotyping can miss certain combinations.

A patient could harbor two or three strains belonging to different bacterial genera that produced the same enzyme, e.g., TEM-3 in *Escherichia coli* and *Klebsiella pneumoniae* from the same urine sample or TEM-3 in *Escherichia coli*, *Salmonella typhimurium*, and *Proteus morganii* from feces and urine samples of the same patient. On the contrary, a patient could harbor two indistinguishable (same species, serotype, and biotype) isolates with different enzymes, e.g., *Klebsiella pneumoniae* strains specifying either a TEM variant or its isozyme differing only at position 261 (phenotype A, Table 4).

An advantage of the oligotyping method is the ability to detect genes encoding enzymes even when they are phenotypically masked by the presence of another β-lactamase. For example, the chromosomal cephalosporinase hyperproduced in certain isolates of *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter agglomerans*, *Enterobacter cloacae*, and *Escherichia coli* did not preclude the detection of bla_{TEM} genes for extended-spectrum enzymes TEM-3, TEM-4, TEM-8, and TEM-12 (data not shown).

DISCUSSION

Until now, the distribution of TEM β-lactamases in gram-negative bacteria has been studied essentially by isoelectric focusing, determination of enzymatic substrate profiles, and determination of the effect of penicillinase inhibitors (17). We developed oligotyping, a fast and sensitive method, to reveal and characterize TEM isozymes in large numbers of clinical isolates. The technique consists of the detection, by colony hybridization with oligonucleotide probes, of point mutations in the structural genes for the enzymes. The heptadecanucleotides (Table 2) were designed to screen for base substitutions at five loci of the bla_{TEM} structural genes, and the technique was applied to 265 clinical isolates of members of the family *Enterobacteriaceae* that were resistant to β-lactams (Table 4). Direct sequencing of amplified bla_{TEM-6}, bla_{TEM-8}, and bla_{TEM-9} genes confirmed the results obtained by oligotyping (15; Goussard et al., unpublished data).

The prevalence of penicillinases TEM-1 and TEM-2 among the 222 strains that produced TEM β-lactamases was similar to that reported by Ouellette et al. (19); TEM-1 alone was the most frequent (15%) and was found in every species studied, whereas TEM-2 alone was rare (1%) and was present only in *Enterobacter cloacae* and *Serratia marces-*

TABLE 5. Predicted and detected combinations of mutations in bla_{TEM} genes enlarging the spectrum of the corresponding enzyme

Combination of mutations		No. of strains
Predicted	Detected	
One mutation		
Lys-102	TEM-6, TEM-16, TEM-17, TEM-18	7
Ser-162	TEM-7, TEM-12	4
Ser-236	TEM-19	4
Thr-235–Lys-237[a]		
Two mutations		
Lys-102, Ser-162	TEM-9	1
Lys-102, Ser-236	TEM-3, TEM-4, TEM-14, TEM-15	146
Lys-102, Thr-235–Lys-237		
Ser-162, Ser-236		
Ser-162, Thr-235–Lys-237	TEM-5	
Ser-236, Thr-235–Lys-237		
Three mutations		
Lys-102, Ser-162, Ser-236	TEM-8	3
Lys-102, Ser-162, Thr-235–Lys-237		
Lys-102, Ser-236, Thr-235–Lys-237		
Ser-162, Ser-236, Thr-235–Lys-237		

[a] Linked mutations corresponding to Thr-235 and Lys-237 were considered as a single entity.

cens. The association of TEM-1 and TEM-2 was also rare (<1%) and occurred only in *Enterobacter cloacae*. Our study confirmed that TEM-3 is the most common enzyme (28); it was detected in every source of strains obtained in France and in 13 bacterial species. Spread of this β-lactamase is due to both bacterial (28) and plasmid (23) epidemics. The already described variants TEM-4, TEM-5, and TEM-7 (Table 1) either remained confined to their original location or were not detected (Table 4). Genes encoding TEM-9 to TEM-19 were found at a single site. Enzymes TEM-6 and TEM-8 were detected in two hospitals.

It has been demonstrated that extended-spectrum mutants can be selected in vitro from penicillinases TEM-1 and TEM-2 (8, 30), and the natural derivative TEM-7 has a single amino acid substitution at position 162 (8). We detected variants that also possessed a single mutation, expanding the substrate range: Lys-102 in TEM-17 and TEM-18, Ser-162 in TEM-12 (TEM-101), and Ser-236 in TEM-19. Furthermore, TEM-17 and TEM-18 differed only at position 37, which indicates their derivation from TEM-1 and TEM-2, respectively. The occurrence in different geographical areas of point mutation alleles indicated that they result from independent genetic events.

Variants with two or three mutations responsible for the enlargement of the enzymatic spectrum could result from sequential mutations. Evidence has been provided for successive isolation, from the same patient, of a strain with an increased resistance toward broad-spectrum cephalosporins that correlated with a change in the pI of the TEM enzyme involved (35). In addition, variants with several mutations are rare and apparently remain confined to their site of isolation: TEM-9 in Great Britain, TEM-11 in Belgium, and TEM-14 and TEM-15 in France. The new TEM enzymes detected in this study were always, but not exclusively, found in *Klebsiella pneumoniae*. The reason for the emergence of the majority of the TEM derivatives in this bacterial species (Table 1) remains unknown.

The predicted and detected combinations of mutations that enlarged the enzymatic substrate range are listed in Table 5. Mutants with one (Lys-102 in TEM-17 and TEM-18, Ser-236 in TEM-19), two (Lys-102 and Ser-162 in TEM-9), or three (Lys-102, Ser-162, and Ser-236 in TEM-8) mutations were first detected in this study. Certain combinations of mutations, in particular those leading to the double substitution Thr-235–Lys-237, have not yet been isolated in nature. Comparison of the mutants selected in vivo with those obtained in vitro with various broad-spectrum cephalosporins or following mutagenesis will be of interest.

Considering the variety of point mutations of TEM and SHV enzymes, one can anticipate the emergence of similar variants in other plasmid-determined β-lactamases such as CARB and OXA.

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LITERATURE CITED

1. **Ambler, R. P.** 1978. Partial amino acid sequence of penicillinase coded by *Escherichia coli* plasmid R6K. Proc. Natl. Acad. Sci. USA **75**:3732–3736.
2. **Arakawa, Y., M. Ohta, N. Kido, Y. Fuji, T. Komatsu, and N. Kato.** 1986. Close evolutionary relationship between the chromosomally encoded β-lactamase gene of *Klebsiella pneumoniae* and the TEM β-lactamase gene mediated by R plasmids. FEBS Lett. **207**:69–74.
3. **Bauernfeind, A., and G. Hörl.** 1987. Novel R-factor borne β-lactamase of *Escherichia coli* conferring resistance to cephalosporins. Infection **15**:257–259.
4. **Brun-Buisson, C., P. Legrand, A. Philippon, F. Montravers, M. Ansquer, and J. Duval.** 1987. Transferable enzymatic resistance to third-generation cephalosporins during nosocomial outbreaks of multiresistant *Klebsiella pneumoniae*. Lancet **ii**:302–306.
5. **Buré, A., P. Legrand, G. Arlet, V. Jarlier, G. Paul, and A. Philippon.** 1988. Dissemination in five french hospitals of *Klebsiella pneumoniae* serotype K25 harbouring a new transferable enzymatic resistance to third generation cephalosporins and aztreonam. Eur. J. Clin. Microbiol. **7**:780–782.
6. **Chen, S. T., and R. C. Clowes.** 1987. Variations between the nucleotide sequence of Tn*1*, Tn*2*, and Tn*3* and expression of β-lactamase in *Pseudomonas aeruginosa* and *Escherichia coli*. J. Bacteriol. **169**:913–916.
7. **Collatz, E., G. Tran Van Nhieu, D. Billot-Klein, R. Williamson, and L. Gutmann.** 1989. Substitution of serine for arginine in position 162 of TEM-type β-lactamases extends the substrate profile of mutant enzymes, TEM-7 and TEM-101, to ceftazidime and aztreonam. Gene **78**:349–354.
8. **Gutmann, L., M. D. Kitzis, D. Billot-Klein, F. Goldstein, G. Tran Van Nhieu, T. Lu, J. Carlet, E. Collatz, and R. Williamson.** 1988. Plasmid-mediated β-lactamase (TEM-7) involved in resistance to ceftazidime and aztreonam. Rev. Infect. Dis. **10**:860–866.
9. **Jarlier, V., M. H. Nicolas, G. Fournier, and A. Philippon.** 1988. Extended broad-spectrum β-lactamases conferring transferable

resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. **10**:867–878.

10. **Johnson, D. A., J. W. Gantsch, J. R. Sportsman, and J. H. Elder.** 1984. Improved technique utilizing non fat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Technol. **1**:3–8.
11. **Kitzis, M. D., D. Billot-Klein, F. W. Golstein, R. Williamson, G. Tran Van Nhieu, J. Carlet, J. F. Acar, and L. Gutmann.** 1988. Dissemination of the novel plasmid-mediated β-lactamase CTX-1, which confers resistance to broad-spectrum cephalosporins, and its inhibition by β-lactamase inhibitors. Antimicrob. Agents Chemother. **32**:9–14.
12. **Kliebe, C., B. A. Nies, J. F. Meyer, R. M. Tolxdorff-Neutzling, and B. Wiedemann.** 1985. Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. Antimicrob. Agents Chemother. **28**:302–307.
13. **Knothe, A., P. Shah, V. Krcmery, M. Antal, and S. Mitsuhashi.** 1983. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection **6**:315–317.
14. **Labia, R. F., F. Le Goffic, and J. Andrillon.** 1974. Etude cinétique de deux β-lactamases responsables d'un même phénotype. Biochimie **56**:1025–1030.
15. **Mabilat, C., S. Goussard, W. Sougakoff, R. C. Spencer, and P. Courvalin.** 1990. Direct sequencing of the amplified structural gene and promoter for the extended-broad-spectrum β-lactamase TEM-9 (RHH-1) of *Klebsiella pneumoniae*. Plasmid **23**: 27–34.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Medeiros, A. A.** 1984. β-Lactamases. Br. Med. Bull. **40**:18–27.
18. **Ouellette, M., L. Bissonnette, and P. H. Roy.** 1987. Precise insertion of antibiotic resistance determinants into Tn*21*-like transposons: nucleotide sequence of the OXA-1 β-lactamase gene. Proc. Natl. Acad. Sci. USA **84**:7378–7382.
19. **Ouellette, M., G. C. Paul, A. M. Philippon, and P. H. Roy.** 1988. Oligonucleotide probes (TEM-1, OXA-1) versus isoelectric focusing in β-lactamase characterization of 114 resistant strains. Antimicrob. Agents Chemother. **32**:397–399.
20. **Ouellette, M., J. J. Rossi, R. Bazin, and P. H. Roy.** 1986. Oligonucleotide probes for the detection of TEM-1 and TEM-2 β-lactamase genes and their transposons. Can. J. Microbiol. **33**:205–211.
21. **Paul, G., M. Barthélémy, A. Philippon, J. Péduzzi, L. Gilly, R. Labia, and P. Névot.** 1988. Immunological comparison of constitutive β-lactamases of gram-negative bacteria by neutralization in zymogram gels: properties of anti-TEM-1 and anti-TEM-2 sera. Ann. Inst. Pasteur (Paris) **139**:435–451.
22. **Paul, G. C., G. Gerbaud, A. Buré, A. M. Philippon, B. Pangon, and P. Courvalin.** 1989. TEM-4, a new plasmid-mediated β-lactamase that hydrolyzes broad-spectrum cephalosporins in a clinical isolate of *Escherichia coli*. Antimicrob. Agents Chemother. **33**:1958–1963.
23. **Petit, A., G. Gerbaud, D. Sirot, P. Courvalin, and J. Sirot.** 1990. Molecular epidemiology of TEM-3 (CTX-1) β-lactamase. Antimicrob. Agents Chemother. **34**:219–224.
24. **Petit, A., D. L. Sirot, C. M. Chanal, J. L. Sirot, R. Labia, G. Gerbaud, and R. A. Cluzel.** 1988. Novel plasmid-mediated β-lactamase in clinical isolates of *Klebsiella pneumoniae* more resistant to ceftazidime than to other broad-spectrum cephalosporins. Antimicrob. Agents Chemother. **32**:626–630.
25. **Quinn, J. P., D. Miyashiro, D. Sahm, R. Flamm, and K. Bush.** 1989. Novel plasmid-mediated β-lactamase (TEM-10) conferring selective resistance to ceftazidime and aztreonam in clinical isolates of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **33**:1451–1456.
26. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**:1350–1354.
27. **Sirot, D., J. Sirot, R. Labia, A. Morand, P. Courvalin, A. Darfeuille-Michaud, R. Perroux, and R. Cluzel.** 1987. Transferable resistance to third generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-1, a novel β-lactamase. J. Antimicrob. Chemother. **20**:323–334.
28. **Sirot, J., C. Chanal, A. Petit, D. Sirot, R. Labia, and G. Gerbaud.** 1988. *Klebsiella pneumoniae* and other *Enterobacteriaceae* producing novel plasmid-mediated beta-lactamases markedly active against third-generation cephalosporins: epidemiologic studies. Rev. Infect. Dis. **10**:850–859.
29. **Sougakoff, W., S. Goussard, and P. Courvalin.** 1988. The TEM-3 β-lactamase, which hydrolyzes broad-spectrum cephalosporins, is derived from the TEM-2 penicillinase by two amino-acid substitutions. FEMS Lett. **56**:343–348.
30. **Sougakoff, W., S. Goussard, G. Gerbaud, and P. Courvalin.** 1988. Plasmid-mediated resistance to third-generation cephalosporins caused by point mutations in TEM-type penicillinase genes. Rev. Infect. Dis. **10**:879–884.
31. **Sougakoff, W., A. Petit, S. Goussard, D. Sirot, A. Buré, and P. Courvalin.** 1989. Characterization of the plasmid genes *blaT-4* and *blaT-5* which encode the broad spectrum β-lactamases TEM-4 and TEM-5 in Enterobacteriaceae. Gene **78**:339–348.
32. **Spencer, R. C., P. F. Wheat, T. G. Winstanley, D. M. Cox, and S. J. Plested.** 1987. Novel β-lactamase in a clinical isolate of *Klebsiella pneumoniae* conferring unusual resistance to β-lactam antibiotics. J. Antimicrob. Chemother. **20**:919–927.
33. **Sutcliffe, J. G.** 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc. Natl. Acad. Sci. USA **75**:3737–3741.
34. **Sykes, R. B., and M. Matthew.** 1976. The β-lactamases of gram-negative bacteria and their role in resistance to β-lactam antibiotics. J. Antimicrob. Chemother. **2**:115–157.
35. **Vuye, A., G. Verschraegen, and G. Claeys.** 1989. Plasmid-mediated β-lactamases in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* resistant to ceftazidime. Antimicrob. Agents Chemother. **33**:757–761.
36. **Wallace, R. B., J. Shaffer, R. H. Murphy, J. Bonner, T. Hirose, and K. Itakura.** 1979. Hybridization of synthetic oligo-deoxyribonucleotides to ΦX174 DNA: the effect of single base pair mismatch. Nucleic Acids Res. **11**:3543–3557.
37. **Wood, W. I., J. Gitschier, L. A. Lasky, and R. M. Lawn.** 1985. Base composition independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc. Natl. Acad. Sci. USA **82**:1585–1588.