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Nucleotide Sequences of CAZ-2, CAZ-6, and CAZ-7 β-Lactamase Genes

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CAZ-2, CAZ-6, and CAZ-7 are plasmid-mediated β-lactamases that are markedly active against ceftazidime. The corresponding structural genes were amplified by the polymerase chain reaction. Nucleotide sequences were determined by direct sequencing of the amplified products. Analysis of the nucleotide and the deduced amino acid sequences showed that CAZ-2, CAZ-6, and CAZ-7 are derived from TEM-2 by three, four, and two amino acid substitutions, respectively. All these substitutions are located at positions 102, 162, 235, 236, and 237 (Sutcliffe numbering), which are known to extend the substrate range of β-lactamases. These substitutions are Lys-102, Ser-162, and Ser-236 in CAZ-2; Lys-102, Ser-162, Thr-235, and Lys-237 in CAZ-6; and Lys-102 and His-162 in CAZ-7. These results indicate that the nucleotide sequence of CAZ-2 is identical to that of TEM-8. The nucleotide sequence of CAZ-7 possesses the two mutations described in TEM-16 by the oligotyping method. In contrast, the combination of mutations encountered in CAZ-6 has not yet been described, and this enzyme was designated TEM-24.

CAZ-2, CAZ-6, and CAZ-7 are extended-spectrum β-lactamases that were isolated from *Klebsiella pneumoniae* at the same hospital (3). DNA-DNA hybridization experiments showed that these enzymes belong to the TEM family. The sequences of many TEM-related extended-spectrum β-lactamases have been published (6) and show that these enzymes differ from TEM-1 or TEM-2 by a few amino acid substitutions (7).

The aim of this study was to determine the nucleotide sequences of the structural genes encoding for CAZ-2, CAZ-6, and CAZ-7 and to compare them with previously published results.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study were *Escherichia coli* CF804, CF1204, and CF1404, which are transconjugants of *K. pneumoniae* CF704 (CAZ-2 producing), CF1104 (CAZ-6 producing), and CF1304 (CAZ-7 producing) (3), respectively.

Oligonucleotides. The oligonucleotides used as primers for amplification and/or sequencing (Table 1) were deblocked by the manufacturer (Institut Pasteur, Centre National de la Recherche Scientifique UA487, Paris, France) and were used without purification.

DNA preparation and PCR. Plasmid DNAs were prepared by the method of Birnboim and Doly (2) and were purified by cesium chloride-ethidium bromide gradient ultracentrifugation.

Single-stranded DNA templates were generated by the polymerase chain reaction (PCR) (10), which was performed with an asymmetric ratio of the two amplification primers (11). One microgram of DNA was amplified (for 60 rounds) in a final volume of 100 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3, 25°C), 1.5 mM $MgCl_2$, 0.01 μg of gelatin per ml, 200 μM (each) deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), 0.2 μM nonlimiting primer, 0.01 μM limiting primer, and 2.5 U of *Taq* polymerase. Samples were overlaid with approximately 100 μl of paraffin oil to prevent evaporation and were then submitted to amplification in a DNA thermal cycler (Perkin-Elmer Cetus Instruments). After a first cycle of denaturation at 94°C for 10 min, annealing at 57°C for 1 min, and polymerization at 72°C for 2 min, 60 cycles were then performed as follows: denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and polymerization at 72°C for 1 min. After the last cycle, the polymerization step was extended by an additional 10 min at 72°C. Amplification reactions were monitored by agarose gel electrophoresis of a 5-μl aliquot.

Purification of amplified DNA. Unincorporated deoxynu-

TABLE 1. Nucleotide sequences of the oligonucleotides used for amplification and/or sequencing reactions

Primer[a]	Sequence	Position[b]
Amplification and sequencing		
A	5′-d[GAAGACGAAAGGGCCTCGTG]-3′	6
B	5′-d[GGTCTGACAGTTACCAATGC]-3′	1079
Sequencing		
C	5′-d[GGGCAAGAGCAACTCGG]-3′	459
D	5′-d[CAGCAATGGCAACAACGTTG]-3′	751
E	5′-d[GAAGCTAGAGTAAGTAG]-3′	810
F	5′-d[TTACTGTCATGCCATCC]-3′	560
G	5′-d[AAGGATCTTACCGCTGT]-3′	373
H	5′-d[TTATTGTCTCATGAGCG]-3′	169

[a] Primers A, C, and D were identical to the leading strand; primers B, E, F, G, and H were identical to the lagging strand.
[b] The numbers correspond to the positions of the first 5′ base of the oligonucleotide by the numbering of Sutcliffe (15).

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CAZ-2
CAZ-6 atttttataggttaatgtcatgataataatggtttcttagacgtcaggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaa 100
                                                                                                          (133)
CAZ-7

CAZ-2
CAZ-6 atacattcaaataagtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagt ATG AGT ATT CAA CAT TTC  193
                                                                                  *                       (226)
CAZ-7

CAZ-2
CAZ-6 CGT GTC GCC CTT ATT CCC TTT TTT GCG GCA TTT TGC CTT CCT GTT TTT GCT CAC CCA GAA ACG CTG GTG AAA GTA  268
                                                                                                          (301)
CAZ-7

CAZ-2
CAZ-6 AAA GAT GCT GAA GAT AAG TTG GGT GCA CGA GTG GGT TAC ATC GAG CTG GAT CTC AAC AGC GGT AAG ATC CTT GAG  343
                                                                                                          (376)
CAZ-7

CAZ-2
CAZ-6 AGT TTT CGC CCC GAA GAA CGT TTT CCA ATG ATG AGC ACT TTT AAA GTT CTG CTA TGT GGT GCG GTA TTA TCC CGT  418
                                                                                                          (451)
CAZ-7

CAZ-2
CAZ-6 GTT GAC GCC GGG CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT TCT CAG AAT GAC TTG GTT AAG TAC TCA CCA GTC  493
                                                                                                          (526)
CAZ-7

CAZ-2
CAZ-6 ACA GAA AAG CAT CTT ACG GAT GGC ATG ACA GTA AGA GAA TTA TGC AGT GCT GCC ATA ACC ATG AGT GAT AAC ACT  568
                                                                                                          (601)
CAZ-7

CAZ-2
CAZ-6 GCG GCC AAC TTA CTT CTG ACA ACG ATC GGA GGA CCG AAG GAG CTA ACC GCT TTT TTG CAC AAC ATG GGG GAT CAT  643
                                                                                                          (676)
CAZ-7

CAZ-2       C
CAZ-6 GTA ACT CGC CTT GAT AGT TGG GAA CCG GAG CTG AAT GAA GCC ATA CCA AAC GAC GAG CGT GAC ACC ACG ATG CCT  718
                                                                                                          (751)
CAZ-7       C             CA

CAZ-2
CAZ-6 GCA GCA ATG GCA ACA ACG TTG CGC AAA CTA TTA ACT GGC GAA CTA CTT ACT CTA GCT TCC CGG CAA CAA TTA ATA  793
                                                                                                          (826)
CAZ-7

CAZ-2
CAZ-6 GAC TGG ATG GAG GCG GAT AAA GTT GCA GGA CCA CTT CTG CGC TCG GCC CTT CCG GCT GGC TGG TTT ATT GCT GAT  868
                                                                                                          (901)
CAZ-7

CAZ-2             G   A   G         A
CAZ-6 AAA TCT GGA ACC GGT AAG CGT GGG TCT CGC GGT ATC ATT GCA GCA CTG GGG CCA GAT GGT AAG CCC TCC CGT ATC  943
                                                                                                          (976)
CAZ-7             G       G         A

CAZ-2
CAZ-6 GTA GTT ATC TAC ACG ACG GGG AGT CAG GCA ACT ATG GAT GAA CGA AAT AGA CAG ATC GCT GAG ATA GGT GCC TCA 1018
                                                                                                         (1051)
CAZ-7

CAZ-2
CAZ-6 CTG ATT AAG CAT TGG TAA ctgtcaga                                                                   1044
                          **                                                                             (1077)
CAZ-7
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FIG. 1. Nucleotide sequences of the CAZ-2, CAZ-6, and CAZ-7 β-lactamase genes. Only nucleotide differences are noted in the CAZ-2 and CAZ-7 sequences. The initiation (*) and the stop (**) codons are indicated. The nucleotide numbers of Sutcliffe (15) are indicated in parentheses.

TABLE 2. Nucleotide and amino acid substitutions among the bla_{TEM} genes that encode CAZ-2, CAZ-6, and CAZ-7 and the bla_{TEM-3} (CTX-1/TEM-3), bla_{TEM-5} (CAZ-1/TEM-5), and bla_{TEM} genes from transposons Tn*1* (TEM-2), Tn*2* (TEM-1), and Tn*3* (TEM-1)

Gene (enzyme)	Substitution at the following nucleotide no. (amino acid position)[a]:														
	226 (6)	263 (19)	317 (37)	346 (46)	436 (76)	512 (102)	604 (132)	682 (158)	692 (162)	693 (162)	911 (235)	914 (236)	917 (237)	925 (239)	990 (261)
bla_{TEM-1A} (TEM-1 [Tn*3*])	C (Phe)	C (Leu)	C (Gln)	A (Glu)	C (Gly)	G (Glu)	G (Ala)	T (Thr)	C (Arg)	G (Arg)	G (Ala)	G (Gly)	G (Glu)	G (Gly)	C (Thr)
bla_{TEM-1B} (TEM-1 [Tn*2*])	T	C	C	A	T	G	T	T	C	G	G	G	G	G	C
bla_{TEM-2} (TEM-2 [Tn*1*])	C	C	A (Lys)	G	T	G	G	C	C	G	G	G	G	A	C
bla_{TEM-3} (CTX-1/TEM-3)	C	C	A (Lys)	G	T	A (Lys)	G	C	C	G	G	A (Ser)	G	A	C
bla_{TEM-5} (CAZ-1/TEM-5)	T	C	C	A	T	G	T	T	A (Ser)	G	A (Thr)	G	A (Lys)	G	C
CAZ-2	C	C	A (Lys)	G	T	A (Lys)	G	C	A (Ser)	G	G	A (Ser)	G	A	C
CAZ-6	C	C	A (Lys)	G	T	A (Lys)	G	T	A (Ser)	G	A (Thr)	G	A (Lys)	G	C
CAZ-7	C	C	A (Lys)	G	T	A (Lys)	G	C	C	A (His)	G	G	G	A	C

[a] Nucleotide numbering is according to Sutcliffe (15). The sequence of bla_{TEM-1A} was determined by Sutcliffe (15), those of bla_{TEM-1B} and bla_{TEM-2} were partially determined by Chen and Clowes (4) and were completed by Goussard and Courvalin (5). The amino acid encoded is indicated when a point mutation leads to an amino acid substitution compared with the sequence of TEM-1 (Tn*3*). Numbering is according to Sutcliffe (15).

cleotide triphosphates (dNTPs) were removed by ethanol precipitation in 2.5 M ammonium acetate and 1 volume of ethanol (−20°C). After 15 min on ice, the sample was spun for 15 min in a microcentrifuge at room temperature and the pellet was washed in 70% ethanol, dried, and resuspended in 21 μl of TE buffer (10 mM Tris HCl, 1 mM EDTA).

Sequencing of PCR-amplified DNA. Seven microliters of the purified PCR product were sequenced by the dideoxy-mediated chain termination method (12) by using the Sequenase Version 2.0 DNA sequencing kit (U.S. Biochemicals, Cleveland, Ohio), as follows: 7 μl of purified DNA, 2 μl of 5× Sequenase buffer (200 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, 250 mM NaCl), and 1 pmol of sequencing primer were mixed in a 10-μl reaction. The mixture was heated at 65°C for 3 min, allowed to cool slowly to room temperature over a period of about 30 min, and then put on ice. Two microliters of Sequenase (1/8 dilution in 10 mM Tris-HCl [pH 7.5], 5 mM dithiothreitol, 0.5 mg of bovine serum albumin per ml), 1 μl of 0.1 M dithiothreitol, 0.5 μl [α-^{35}S]dATP, and 2 μl of the labeling mixture containing dGTP, dCTP, and dTTP (1.5 μM each) were added to the annealed template-primer and incubation was allowed to continue for 3 min at room temperature. A total of 3.5 μl of this mixture was distributed in four tubes, with each tube containing 2.5 μl of the ddGTP, ddATP, ddTTP, and ddCTP termination mixtures, respectively. The tubes were incubated at 37°C for 5 min. Four microliters of stop solution was then added. After denaturation at 85°C for 2 min, the product of the sequencing reaction (3 to 5 μl) was resolved by electrophoresis on 6% polyacrylamide–7 M urea–1× Tris-borate-EDTA vertical gels (40 by 30 by 0.04 cm). Gels were soaked for 15 min in 5% acetic acid–15% methanol to remove the urea, dried, and autoradiographed for 24 to 48 h at room temperature.

Nucleotide sequence accession numbers. The CAZ-2, CAZ-6, and CAZ-7 β-lactamase sequences have been submitted to GenBank and have been assigned the accession numbers X65252, X65253, and X65254, respectively.

TABLE 3. Amino acid substitutions in CAZ-2, CAZ-6, and CAZ-7 compared with those in other TEM derivative β-lactamases

β-Lactamase	pI	Substitution at amino acid position[a]:						Reference or source
		37	102	162	235	236	237	
TEM-1	5.4	Gln	Glu	Arg	Ala	Gly	Glu	15
TEM-2	5.6	Lys						1
TEM-3 (CTX-1)	6.3	Lys	Lys			Ser		13
TEM-5 (CAZ-1)	5.55			Ser	Thr		Lys	14
TEM-6	5.85		Lys	His				6
TEM-8	5.9	Lys	Lys	Ser		Ser		9
TEM-16	6.3	Lys	Lys	His				8
CAZ-2	5.9	Lys	Lys	Ser		Ser		This study
CAZ-6	6.5	Lys	Lys	Ser	Thr		Lys	This study
CAZ-7	6.3	Lys	Lys	His				This study

[a] Amino acid residues are numbered as described by Sutcliffe (15) for TEM-1. For the other sequences, only amino acids which differed from those of TEM-1 are indicated.

RESULTS AND DISCUSSION

The nucleotide sequences of the amplified fragments are shown in Fig. 1. Open reading frames of 858 nucleotides corresponding to the bla_{TEM} genes that encode the CAZ-2, CAZ-6, and CAZ-7 enzymes were identified. The nucleotide substitutions relative to those in other bla_{TEM} genes are listed in Table 2. Analysis of the nucleotide and the deduced amino acid sequences showed that CAZ-2, CAZ-6, and CAZ-7 differ from TEM-2 by three, four, and two amino acid substitutions, respectively. All these substitutions are located at positions 102, 162, 235, 236, and 237, which are known to extend the substrate range of β-lactamases. The amino acid alterations described for other extended-spectrum β-lactamases in the TEM family are summarized in Table 3.

The nucleotide sequence of CAZ-2 is identical to that of TEM-8 (9). These two enzymes differ from TEM-2 by the following three amino acid substitutions: Glu → Lys 102, Arg → Ser 162, and Gly → Ser 236. These results are not surprising since the two enzymes possess the same pI (5.9) and confer the same resistance phenotype.

The sequence of the gene that encodes CAZ-2 is very close to that of the bla_{TEM-3} gene that encodes CTX-1 (13); the two genes differ only at position 692 (Sutcliffe [15] numbering; an adenine in CAZ-2 gene versus a cytosine in bla_{TEM-3}). Since the first isolate of *K. pneumoniae* that was found to produce CAZ-2 was detected after ceftazidime

treatment in a patient who had previously harbored CTX-1-producing strains, we suppose that CAZ-2 could have been derived from CTX-1 by one point mutation, leading to the substitution Arg → Ser-162.

The nucleotide sequence of CAZ-7 differs from that of TEM-2 by two point mutations localized at positions 512 (adenine in CAZ-7 versus guanosine in TEM-2) and 693 (adenine in CAZ-7 versus guanosine in TEM-2). These two mutations led to the substitutions Glu → Lys-102 and Arg → His-162.

The amino acid sequence of CAZ-7 is very close to that of TEM-6 (6). These two enzymes differ only at position 37: Gln in TEM-6 (TEM-1 derived) versus Lys in CAZ-7 (TEM-2 derived). TEM-6 and CAZ-7 possess the same amino acid substitutions involved in hydrolysis of extended-spectrum cephalosporins. However, they differ by their pIs, as follows: 5.85 for TEM-6 versus 6.3 for CAZ-7.

The two substitutions Lys-102 and His-162 were also detected in TEM-16 by the oligotyping method (8). CAZ-7 and TEM-16 possess the same pI (6.3). However it is difficult to establish the identities of these two enzymes, since the substrate profile of TEM-16 and the complete sequence of bla_{TEM-16} are not available.

The combination of substitutions Lys-102, Ser-162, Thr-235, and Lys-237 encountered in CAZ-6 has not yet been described. Therefore, we propose the designation TEM-24 for CAZ-6. Analysis of nucleotide sequences (Table 2) shows that the gene bla_{TEM-24} (encoding CAZ-6/TEM-24) is identical to gene bla_{TEM-3} (encoding CTX-1/TEM-3) (13) in its initial part and is identical to bla_{TEM-5} (encoding CAZ-1/TEM-5) (14) in its final part.

It is interesting that the first isolate that was found to produce CAZ-6 was detected in a patient who had previously harbored CTX-1/TEM-3- and CAZ-1/TEM-5-producing strains. These observations suggest that CAZ-6 could be the result of recombination of bla_{TEM-3} and bla_{TEM-5} (likely located between positions 604 and 682).

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