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TEM-109 (CMT-5), a Natural Complex Mutant of TEM-1 β-Lactamase Combining the Amino Acid Substitutions of TEM-6 and TEM-33 (IRT-5)†

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***Escherichia coli* CF349 exhibited a complex β-lactam resistance phenotype, including resistance to amoxicillin and ticarcillin alone and in combination with clavulanate and to some extended-spectrum cephalosporins. The double-disk synergy test was positive. CF349 harbored an 85-kb conjugative plasmid which encoded a β-lactamase of pI 5.9. The corresponding *bla* gene was identified by PCR and sequencing as a bla_{TEM} gene. The deduced protein sequence revealed a new complex mutant of TEM-1 β-lactamase designated TEM-109 (CMT-5). TEM-109 contained both the substitutions Glu104Lys and Arg164His of the expanded-spectrum β-lactamase (ESBL) TEM-6 and Met69Leu of the inhibitor-resistant TEM-33 (IRT-5). TEM-109 exhibited hydrolytic activity against ceftazidime similar to that of TEM-6 (k_{cat}, 56 s^{-1} and 105 s^{-1}, respectively; K_m values, 226 and 247 μM, respectively). The 50% inhibitory concentrations of clavulanate and tazobactam (0.13 μM and 0.27 μM, respectively) were 5- to 10-fold higher for TEM-109 than for TEM-6 (0.01 and 0.06 μM, respectively) but were almost 10-fold lower than those for TEM-33. The characterization of this novel CMT, which exhibits a low level of resistance to inhibitors, highlights the emergence of this new ESBL type.**

The most prevalent mechanism of resistance to β-lactam antibiotics in members of the family *Enterobacteriaceae* is the production of β-lactamases belonging to Bush group 2b (6, 21). These enzymes are able to inactivate penicillins and narrow-spectrum cephalosporins before they can reach their target.

Extended-spectrum β-lactamases (ESBLs) were isolated first in Europe and then worldwide shortly after the introduction of oxyimino cephalosporins (28, 30). According to the structural classification of Ambler et al. (1) and the functional classification of Bush and Jacoby (6), these first ESBLs were class A enzymes of the 2be group that arose subsequent to a few number of amino acid substitutions from the common plasmid-mediated TEM and SHV-1 β-lactamases. These enzymes confer resistance to penicillins, oxyimino cephalosporins, and aztreonam and are susceptible to β-lactam inhibitors.

The use of β-lactamase inhibitors has also been followed by the emergence of resistant clinical isolates, which overproduce TEM-type β-lactamases (18) or which produce inhibitor-resistant TEM variants (IRTs) (3). As was the case for the ESBLs, IRTs arose from the common plasmid-mediated TEM and SHV-1 penicillinases subsequent to a few amino acid substitutions. These substitutions conferred resistance to inhibitors but not the ability to hydrolyze oxyimino β-lactams.

A new subgroup of TEM- and SHV-type β-lactamases which harbors both mutations conferring extended-spectrum activity and resistance to inhibitors has emerged since the end of the 1990s in different species of the family *Enterobacteriaceae*: *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterobacter aerogenes*. Four enzymes are derived from TEM-type enzymes and were designated complex mutant TEM (CMT-1 to CMT-4) (10, 23, 24, 29). Another complex mutant derived from SHV-1 has been identified: SHV-10 (25). In 2001, we isolated *E. coli* CF349, a clinical isolate resistant to amoxicillin and ticarcillin alone and in combination with clavulanate and also to some extended-spectrum cephalosporins. The aim of this work was to characterize the β-lactamases involved in this resistance phenotype.

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† This paper is devoted to the memory of Catherine Chanal.

MATERIALS AND METHODS

Strains and plasmids. The strains used in this work included *E. coli* CF349, *E. coli* CF0051 producing TEM-33 (12), *E. coli* HB251 producing TEM-6 (2, 11), and *E. coli* CF001 producing TEM-1 (12). *E. coli* DH5α (Novagen, Darmstadt, Germany) and *E. coli* BL21(DE3) (Novagen, Darmstadt, Germany) were used for the cloning experiments, and *E. coli* C600 was used for the mating-out assays. The plasmid pBK-CMV (Stratagene, Amsterdam, The Netherlands) was used for the initial cloning experiments, and a modified pET9a plasmid (20) was used for the overexpression of the β-lactamase-encoding genes.

Susceptibility to β-lactams. Antibiotic-containing disks were used for antibiotic susceptibility testing by the disk diffusion assay (Sanofi-Diagnostics Pasteur, Marnes la Coquette, France). The double-disk synergy test was performed as described previously (13). MICs were determined by a microdilution method on Mueller-Hinton agar (Sanofi Diagnostics Pasteur) with an inoculum of 10^4 CFU per spot. The antibiotics were provided as powders by GlaxoSmithKline (amoxicillin, ticarcillin, cefuroxime, ceftazidime, and clavulanic acid), Lederle Laboratories (piperacillin and tazobactam), Eli Lilly (cephalothin), Roussel-Uclaf (cefotaxime and cefpirome), Bristol-Myers Squibb (aztreonam and cefepime), and Merck Sharp & Dohme-Chibret (cefoxitin and imipenem).

Isoelectric focusing. Isoelectric focusing was performed with polyacrylamide gels containing ampholines with a pH range of 3.5 to 10.0, as described previously (29). β-Lactamases with known pIs were used as standards: TEM-33 (pI 5.2), TEM-1 (pI 5.4), TEM-2 (pI 5.6), and TEM-6 (pI 5.9).

Mating-out experiment. Direct transfer of plasmids coding for resistance genes was performed by mating donor strains with in vitro-obtained rifampin-resistant mutants of *E. coli* C600 as the recipient strain at 37°C in solid Mueller-Hinton medium (26). Transconjugants were selected on agar containing rifampin (300 μg/ml) and ticarcillin (32 μg/ml).

Plasmid content analysis. Plasmid DNAs were extracted from the transconjugants by the method of Kado and Liu (14). The plasmid size was determined

TABLE 1. MICs of β-lactams for *E. coli* CF349; transconjugant *E. coli* C600(pCF349); and TEM-109-, TEM-6-, TEM-33-, and TEM-1-producing *E. coli* DH5α and *E. coli* DH5α(pBK-CMV)

β-Lactam	MIC (μg/ml) for the following strains (TEM promoter[a]):						
	E. coli CF349 (P_a/P_b)	*E. coli* C600 (pCF349) (P_a/P_b)	*E. coli* DH5α (pBK-TEM-109) (P_a/P_b)	*E. coli* DH5α (pBK-TEM-6) (P_6)	*E. coli* DH5α (pBK-TEM-33) (P_3)	*E. coli* DH5α (pBK-TEM-1) (P_3)	*E. coli* DH5α (pBK-CMV)
Amoxicillin	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	4
Amoxicillin-CLA[b]	128	128	512	32	512	16	4
Ticarcillin	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	2
Ticarcillin-CLA	256	256	256	32	128	32	2
Piperacillin	128	128	512	256	512	512	2
Piperacillin-TZB[c]	4	4	4	2	4	2	2
Cephalothin	16	16	8	16	4	4	4
Cefuroxime	8	8	16	8	4	4	4
Cefoxitin	8	8	4	4	4	4	4
Cefotaxime	0.12	0.12	0.5	0.5	0.06	0.06	0.06
Cefotaxime-CLA	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Ceftazidime	64	64	256	256	0.12	0.12	0.12
Ceftazidime-CLA	2	2	8	2	0.12	0.12	0.12
Aztreonam	16	16	64	64	0.06	0.12	0.12
Aztreonam-CLA	2	2	1	0.25	0.12	0.12	0.12
Cefepime	0.5	0.5	1	1	0.06	0.06	<0.06
Cefpirome	0.5	0.5	1	1	0.06	0.06	<0.06
Imipenem	0.25	0.25	0.25	0.25	0.25	0.25	0.25

[a] Specific activities of crude extracts from *E. coli* DH5α clones (μmol of benzylpenicillin/min/mg of proteins): TEM-109, 5.2; TEM-6, 9.7; TEM-33, 0.7; TEM, 1.6.
[b] CLA, clavulanic acid at 2 μg/ml.
[c] TZB, tazobactam at 4 μg/ml.

by comparison with those of plasmids Rsa (39 kb), TP114 (61 kb), pCFF04 (85 kb), and pCFF14 (180 kb).

Genotyping. The clinical isolates of *E. coli* CF349 were compared by enterobacterial repetitive intergenic consensus sequence PCR (ERIC2-PCR) and ribotyping, as described previously (9, 33).

Cloning experiments. Recombinant DNA manipulation and transformations were performed as described by Sambrook et al. (26). T4 DNA ligase and proofreading *Taq* polymerase were purchased from Appligène (Oncor, Illkirch, France). The TEM-encoding genes, including their promoters, were amplified by PCR with primers TEM-A (5′-TAA AAT TCT TGA AGA CG-3′) and TEM-B2 (5′-TCT GAC AGT TAC CAA TGC-3′) and cloned into SmaI restriction site of pBK-CMV plasmid. The correct orientation of the insert was checked by PCR with the primers TEM-A and pBK-CMV2′ (5′-AAT TGG GTA CAC TTA CCT GGT ACC C-3′). The TEM-encoding genes were also amplified with the primers NdeI-TEM-A (5′-GGA ATT CCA TAT GAG TAT TCA ACA TTT CCG-3′) and NotI-TEM-B (5′-ATA GTT TAG CGG CCG CTT AAT GCT TAA TCA GTG AG-3′), which included restriction sites for the enzymes NdeI and NotI, respectively. The PCR products were digested by these enzymes and ligated into the corresponding restriction sites of a modified pET9a plasmid. The plasmids derived from pBK-CMV and pET9a (pBK-TEM-1, pET9-TEM-1, pBK-TEM-33, pET9-TEM-33, pBK-TEM-6, pET9-TEM-6, pBK-TEM-109, and pET9-TEM-109) were transformed after sequencing of the control into *E. coli* strains DH5α and BL21(DE3), respectively. *E. coli* transformants were selected on Mueller-Hinton agar supplemented with 30 μg kanamycin and 0.5 μg ceftazidime.

Sequencing of DNA amplified by PCR. Direct sequencing of both strands of the PCR products was performed by the dideoxy chain termination with an Applied Biosystems sequencer (ABI 377) (27). The nucleotide and deduced protein sequences were analyzed with software available at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The ClustalW program (http://infobiogen.fr) was used to align the multiple protein sequences (32).

Overexpression and purification of β-lactamases. TEM-producing *E. coli* BL21(DE3) were used to overproduce the β-lactamases, as described previously (8). The strains were cultured in 2x YT broth (Qbiogene, Irvine, Calif.) containing kanamycin (30 μg/ml) and 0.1 mM isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Co., St. Louis, Mo.) The bacteria were disrupted by sonication. The supernatant was loaded onto a Q Sepharose column (10 ml; Amersham Pharmacia Biotech.) equilibrated with 20 mM Tris-HCl (pH 7.0). The bound proteins were eluted with a linear NaCl gradient (0 to 500 mM). The β-lactamase-containing elution peak was loaded onto a Superose 12 column (3.2 by 30 cm; Amersham Pharmacia Biotech.) and eluted with the buffer 20 mM Tris-HCl—100 mM NaCl (pH 7.0). The β-lactamase-containing elution peak was dialyzed against 100 mM NaCl, concentrated by ultrafiltration, and stored at −20°C until use. The total protein concentration was estimated by the Bio-Rad (Richmond, Calif.) protein assay, with bovine serum albumin (Sigma Chemical Co.) used as a standard. The level of purity was estimated to be >97% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4, 17).

Determination of β-lactamase kinetic parameters k_{cat} and K_m and IC_{50}s. The Michaelis constant (K_m) and catalytic activity (k_{cat}) were determined with purified extracts by a computerized microacidimetric method (16). The 50% inhibitory concentrations (IC_{50}s) were determined for clavulanic acid and tazobactam, as described previously, with 100 μM benzylpenicillin as the reporter substrate (4).

Nucleotide sequence accession number. The nucleotide sequence of the $bla_{TEM-109}$ gene has been assigned accession number AY628175 in the GenBank database.

RESULTS AND DISCUSSION

Clinical isolates. Two *E. coli* isolates were collected from stool specimens of patients admitted in the intensive care unit of the teaching hospital of Clermont-Ferrand in 2001 and were selected on the basis of their ability to grow on agar plates containing 4 μg/ml ceftazidime. The two *E. coli* isolates had the same resistance phenotype and were genotypically identical by ribotyping and ERIC2-PCR (data not shown). These isolates, designated CF349, presented high levels of resistance to penicillins and ceftazidime and low levels of resistance to cephalothin and aztreonam. MICs of methoxyimino cephalosporins were in the susceptible range but were significantly higher than those obtained for *E. coli* DH5α, which did not produce a TEM-type β-lactamase. In contrast, the MICs of cefoxitin and imipenem were identical for CF349 and DH5α. If tazobactam restored the activity of piperacillin, clavulanic acid was less effective in decreasing the MICs of amoxicillin, ticarcillin, ceftazidime, and aztreonam (Table 1). However, the synergy test was positive with ceftazidime.

Isoelectric focusing and transfer experiment. *E. coli* CF349 produced two β-lactamases with pI values of 5.4 and 5.9. The genes encoding resistance to β-lactam antibiotics were transferred by conjugation to rifampin-resistant *E. coli* C600. Two types of transconjugants were obtained: the first produced only the β-lactamase of pI 5.4, and the second produced only the β-lactamase of pI 5.9. Plasmid content analysis of the second transconjugant showed the transfer of an 85-kb plasmid conferring a phenotype of resistance to β-lactams, tobramycin, gentamicin, and netilmicin, similar to that of the clinical strain CF349.

PCR experiments and DNA sequencing. Analysis of the bla_{TEM} nucleic acid sequences from the transconjugants revealed a TEM-1-encoding gene corresponding to the β-lactamase of pI 5.4 and a new bla_{TEM}-type gene called $bla_{TEM-109}$ corresponding to the β-lactamase of pI 5.9. In its promoter zone, $bla_{TEM-109}$ differed from bla_{TEM-1a} by the substitution C→T at position 32, according to the numbering of Sutcliffe (31), which leads to the strong overlapping promoter P_a/P_b (18, 19). The sequence of $bla_{TEM-109}$ showed a pattern of silent mutations identical to those of bla_{TEM-1b}: A175G, C226T, C436T, and G604T. In the coding region, $bla_{TEM-109}$ differed from bla_{TEM-1b} by three point mutations: the nucleotide change A→C at position 407, which led to the amino acid substitution Met→Leu at position 69, and the nucleotide change G→A at positions 512 and 693, which led to the amino acid substitutions Glu→Lys at position 104 and Arg→His at position 164 (1). This enzyme harbors the substitutions described in the IRT-5/TEM-33 (Leu-69) and in the ESBL enzyme TEM-6 (Lys-104 and the His-164). This is the fifth member of the complex mutant TEM group (and therefore is also indicated CMT-5) and the second example of the production of such an enzyme by *E. coli*.

MICs for TEM-6-, TEM-33-, and TEM-109-producing transformants. TEM-109-, TEM-6-, and TEM-33-producing *E. coli* DH5α transformants were used to compare these related TEM-encoding genes (Table 1). TEM-109- and TEM-6-producing *E. coli* DH5α exhibited the same high level of resistance to penicillins and carboxypropyl oxyimino β-lactams. The MICs of cephalothin, cefotaxime, cefepime, and cefpirome were also identical for these two strains and increased slightly in comparison with those for *E. coli* DH5α. In contrast, the MICs of the combinations of β-lactams and clavulanic acid were significantly higher for TEM-109-producing *E. coli* DH5α than for TEM-6-producing *E. coli* DH5α.

The MICs of the combinations amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, and piperacillin-tazobactam were comparable for TEM-109- and TEM-33-producing *E. coli* DH5α.

Enzymatic parameters. The kinetic parameters for TEM-1, TEM-6, TEM-33, and TEM-109 were determined under the same experimental conditions (Table 2). The kinetic constants obtained for TEM-1 were similar to those previously determined in another study (29).

The k_{cat} values of TEM-109 were 3- to 20-fold lower for penicillins than those of TEM-1. These values (k_{cat}, 42 to 171 s^{-1}) were more closely related to those of TEM-6 (k_{cat}, 23 to 134 s^{-1}). The K_m values for penicillins were higher for TEM-109 (20 to 64 μM) and TEM-1 (34 to 55 μM) than for TEM-6 (2 to 18 μM). Overall, the catalytic efficiency of TEM-109 against penicillins (k_{cat}/K_m, 2 to 8 $\mu M^{-1}\ s^{-1}$) was more

TABLE 2. Kinetic parameters of β-lactamases TEM-129, TEM-6, TEM-33, and TEM-1

β-Lactam	TEM-109			TEM-6			TEM-33			TEM-1		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
Benzylpenicillin	171 ± 2	20 ± 2	8	134 ± 9	10 ± 1	13	150 ± 10	15 ± 1	10	1,500 ± 120	34 ± 4	44
Amoxicillin	66 ± 3	15 ± 1	4	31 ± 2	8 ± 1	4	111 ± 8	23 ± 3	5	1,125 ± 150	15 ± 2	77
Ticarcillin	42 ± 2	14 ± 2	3	23 ± 3	2 ± 0.3	14	2 ± 0.3	7 ± 2	0.2	135 ± 10	36 ± 4	4
Piperacillin	152 ± 10	64 ± 4	2	107 ± 8	18 ± 2	6	83 ± 11	15 ± 2	6	1,250 ± 70	55 ± 6	23
Cephalothin	15 ± 1	181 ± 12	0.1	74 ± 9	247 ± 12	0.3	5 ± 1	317 ± 20	0.01	165 ± 15	242 ± 12	0.7
Cefuroxime	22 ± 3	93 ± 7	0.2	4 ± 0.3	44 ± 5	0.1	<0.1	ND[a]		<0.1	ND	
Aztreonam	38 ± 5	130 ± 15	0.3	22 ± 3	51 ± 7	0.4	<0.1	ND		<0.1	ND	
Ceftazidime	56 ± 8	226 ± 18	0.2	105 ± 13	247 ± 24	0.4	<0.1	ND		<0.1	ND	
Cefotaxime	21 ± 3	330 ± 26	0.1	9 ± 1	71 ± 9	0.1	<0.1	ND		<0.1	ND	

[a] ND, not determined.

closely related to that of TEM-6 (k_{cat}/K_m, 4 to 14 μM^{-1} s^{-1}) than to that of TEM-1 (k_{cat}/K_m, 4 to 77 μM^{-1} s^{-1}). The same observations were made for TEM-33 (k_{cat}/K_m, 0.2 to 10 μM^{-1} s^{-1}).

The kinetic constants of TEM-109 and TEM-6 against cephalosporins clearly differed from those of TEM-1 and TEM-33. TEM-33 was 10- to 70-fold less efficient against cephalothin than TEM-109, TEM-6, and TEM-1. In addition, only the TEM-109 and TEM-6 enzymes exhibited significant hydrolytic activity against oxyimino β-lactams (k_{cat}, 4 to 105 versus <0.1 s^{-1}). Only slight differences in k_{cat} and K_m values were observed for these substrates between these two enzymes. The catalytic efficiencies of TEM-109 and TEM-6 against oxyimino β-lactams were therefore globally similar (k_{cat}/K_m, 0.1 to 0.4 μM^{-1} s^{-1}). This activity of TEM-109 and TEM-6 against oxyimino β-lactams is explained by the substitutions Glu104Lys and Arg164His.

The presence of lysine at position 104 slightly enhances oxyimino cephalosporin hydrolysis (15) but seems insufficient alone to confer a high level of resistance to extended-spectrum cephalosporins. This substitution is often associated with an additional amino acid substitution at position 238 or 164 (15). TEM-109 harbors the substitution Arg164His, which confers improved catalytic efficiency against oxyimino β-lactams such as ceftazidime and aztreonam. Position 164 is located in a wall of the binding site, the Ω loop, which limits the accommodation of large β-lactams such as oxyimino β-lactams. The substitution at position 164 leads to the flexibility of the Ω loop (15, 34), which can favor the accommodation of the acyl-amide substitutes of ceftazidime and aztreonam, as observed in the crystal structure of TEM-64 (34).

The IC_{50} of clavulanic acid (0.13 ± 0.01 μM) for TEM-109 was 10-fold higher than that of TEM-6 (0.01 ± 0.001 μM), slightly higher than that of TEM-1 (0.08 ± 0.01 μM), and 10-fold lower than that of TEM-33 (1.9 ± 0.1 μM). Similarly, tazobactam was less efficient against TEM-109 (0.27 ± 0.02 μM) than against TEM-6 (0.06 ± 0.004 μM) and TEM-1 (0.13 ± 0.01 μM) and more efficient than against TEM-33 (2.3 ± 0.1 μM). Compared to other CMT enzymes, TEM-109 had lower resistance to clavulanic acid than CMT-1 (29) or CMT-4 (24). CMT-1 and CMT-4 harbor highly efficient IRT-type mutations or associations of mutations. TEM-109 harbors the substitution Met69Leu of TEM-33. TEM-33, which harbors only this substitution, is one of the IRT-type enzymes that is the least resistant to clavulanic acid (5, 7, 22). Residue Leu at position 69 enhances clavulanic acid resistance by increasing the turnover against inhibitors (22). However, no structural evidence explains the behavior induced by Leu69 (22). The weak efficiency of residue Leu69 partially explains the level of resistance of TEM-109 to inhibitors. Despite the slight inhibitor resistance of TEM-109, TEM-109-producing *E. coli* DH5α harbored MICs of penicillins and clavulanic acid in association similar to those of TEM-33-producing *E. coli* DH5α. Its resistance to β-lactamase inhibitors was probably enhanced by the presence of the overlapping promoter *Pa*/*Pb*, which is responsible for the overproduction of TEM enzymes (18, 19), as assessed by their specific activities (Table 1).

The kinetic constants for previous CMT enzymes suggest that the combination of ESBL and IRT mutations have antagonist effects, which conferred to the CMT enzymes kinetic parameters against expanded-spectrum generation cephalosporins lower than those observed with the parental ESBL enzyme (10, 23–25, 29). TEM-109 differed from previously reported CMT enzymes by kinetic parameters almost identical to those of its parental ESBL, TEM-6. The presence of a leucine at position 69 may be responsible for the persistence of the catalytic efficiency of its parental ESBL, TEM-6. Different work suggests that the slight modification of the active site due to the Met69Leu substitution modified only the hydrolysis of small β-lactams, such as clavulanic acid or tazobactam (22). If ESBL mutations Glu104Lys and Arg164His decrease the stability of the enzyme (−1.94 kcal/mol) (34), Wang et al. have also shown that residue Leu69 enhances enzyme stability (1 kcal/mol), unlike residues Ile69 and Val69 (35). Residue Leu69 may partially restore the stability and therefore contribute to the low discrepancies of oxyimino β-lactam MICs between TEM-109 and TEM-6.

In conclusion, we observed *E. coli* isolates resistant to oxyimino β-lactams and penicillins and clavulanic acid in association. The resistance was due to a bla_{TEM} gene containing a strong promoter P_a/P_b and producing the novel enzyme TEM-109 (CMT-5). This enzyme harbored ESBL mutations Glu104Lys and Arg164His in association with the weak and stabilizing IRT mutation Met69Leu. The association of these mutations with a strong promoter may constitute a good compromise that allows extended-spectrum activity, resistance to inhibitors, and stability.

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