ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2009, p. 4518–4521 Vol. 53, No. 10
0066-4804/09/$08.00+0 doi:10.1128/AAC.00461-09

New Variant of CTX-M-Type Extended-Spectrum β-Lactamases, CTX-M-71, with a Gly238Cys Substitution in a *Klebsiella pneumoniae* Isolate from Bulgaria▽

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Received 7 April 2009/Returned for modification 30 May 2009/Accepted 12 July 2009

A single *Klebsiella pneumoniae* strain isolated in a Bulgarian hospital was found to produce CTX-M-71, a new CTX-M variant characterized by one amino acid substitution from glycine to cysteine at position 238 in comparison to CTX-M-15. This exchange decreased the hydrolytic activity of the β-lactamase for cefotaxime, ceftazidime, and cefepime.

Since the first reports on CTX-M-type extended-spectrum β-lactamases (ESBLs) in the late 1980s and early 1990s (2, 4, 13), their number has increased to 89 (http://www.lahey.org/studies [last accessed in June 2009]) and they have become the most prevalent ESBL-type worldwide (6).

Several amino acid residues of CTX-M β-lactamases have been investigated with respect to their influence on hydrolytic activity. Asn104, Asn132, and Ser237 are thought to fix the cefotaxime substrate within the binding site (5). Arg276 appears to be important for the hydrolysis of cefotaxime, and the substitutions Asp240Gly and Pro167Ser improve the hydrolytic activity of CTX-M enzymes for ceftazidime (5, 17).

We analyzed a *Klebsiella pneumoniae* isolate producing a new CTX-M variant, CTX-M-71, with one amino acid substitution (Gly238Cys) in comparison to CTX-M-15.

K. pneumoniae AH24-270 was isolated in September 2003 from the urine of a 29-year-old male patient at the Alexandrovska University Hospital in Sofia, Bulgaria. The patient had been hospitalized since June 2003 due to cranial brain trauma, aspiration pneumonia, and respiratory dysfunction. Antibiotic treatment prior to isolation of the strain included penicillin, metronidazole, amikacin, vancomycin, meropenem, ceftazidime, and piperacillin-tazobactam.

The sequencing of the $bla_{\text{CTX-M}}$ gene of *K. pneumoniae* AH24-270, by using the oligonucleotides CTX-M-1/P1c (5′-TCGTCTCTTCCAGAATAAGG-3′) and CTX-M-1/P2c (5′-AAGGAGAACCAGGAACCACG-3′), revealed one nucleotide exchange from G to T at open reading frame position 721, causing an amino acid substitution from glycine to cysteine at position 238 in comparison to CTX-M-15 (Ambler numbering [1]). This new CTX-M variant was named CTX-M-71. So far, only one other natural CTX-M β-lactamase, namely, CTX-M-34 (accession no. AY515297), which also harbored a cysteine at position 238 was described (15). All other CTX-M variants carry a glycine at that position. However, Shimizu-Ibuka et al. introduced in vitro the Gly238Cys exchange in Toho-1 (CTX-M-44) (19).

Conjugative plasmid transfer, performed as described previously (11), located $bla_{\text{CTX-M-71}}$ on a transferable plasmid together with determinants for resistance to tobramycin, gentamicin, and tetracycline (data not shown).

The MICs, determined by an agar dilution procedure following Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines (16), are shown in Table 1. Interestingly, *K. pneumoniae* AH24-270 was resistant to ertapenem and had an elevated MIC for meropenem but was susceptible to imipenem. The MICs for meropenem and ertapenem, but not for imipenem and faropenem, were lowered in combination with clavulanic acid. The transconjugant showed no elevated MICs for carbapenems.

The isoelectric focusing (IEF) of β-lactamases was described previously (3, 14). After IEF, a bioassay was used to determine the hydrolytic activity of individual β-lactamase bands (3). For both the *K. pneumoniae* AH24-270 wild-type strain and its transconjugant, the IEF of crude homogenates revealed β-lactamases with pIs of 5.4, 7.4, and 8.8. The enzymes focusing at pI 5.4 and 7.4 did not hydrolyze cefotaxime and ceftazidime. While the first one was assumed to be TEM-1, the second one was identified by partial sequencing as OXA-1 (data not shown). Both enzymes are commonly coencoded on $bla_{\text{CTX-M-15}}$-harboring plasmids (12). The enzyme with the pI of 8.8 hydrolyzed cefotaxime and corresponded to CTX-M-71. A fourth β-lactamase focusing at a pI of 7.6 without the hydrolysis of cefotaxime or ceftazidime was produced by the wild-type strain only and most probably corresponded to the chromosomal SHV-type enzyme. Due to the elevated MIC of the wild-type strain for meropenem, a bioassay with meropenem as the substrate was conducted and revealed slight hydrolysis by the β-lactamase with a pI of 8.8 (CTX-M-71) for both the wild type and transconjugant.

For comparison, CTX-M-71 and CTX-M-15 were expressed in an isogenic background. Therefore, the β-lactamase genes of *K. pneumoniae* AH24-270 and *K. pneumoniae* AH27 (a

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▽ Published ahead of print on 20 July 2009.

TABLE 1. Antimicrobial susceptibilities of wild-type, transconjugant, and transformant strains

Antibiotic[a]	MIC (μg/ml) for[b]:					
	K. pneumoniae AH24-270 (CTX-M-71)	*E. coli* K-12		*E. coli* DH5α		
		R^+ (CTX-M-71)	R^-	pBC-CTX-M-71	pBC-CTX-M-15	Host strain
Amoxicillin	>512	>512	2	>512	>512	4
Amoxicillin-clavulanic acid	>512	64	2	8	8	2
Piperacillin	>256	>256	1	256	>256	2
Piperacillin-tazobactam	>256	16	1	2	2	1
Cefuroxime	>512	256	2	512	>512	4
Ceftazidime	16	4	0.06	8	64	0.13
Ceftazidime-clavulanic acid	4	0.25	0.06	0.25	0.25	0.06
Cefotaxime	64	4	0.03	16	>256	0.03
Cefotaxime-clavulanic acid	2	0.06	0.016	0.03	0.06	0.016
Cefepime	128	2	0.016	4	16	0.016
Cefoxitin	64	2	2	4	4	4
Aztreonam	128	8	0.03	8	64	0.03
Meropenem	4	0.03	0.03	0.03	0.03	0.03
Meropenem-clavulanic acid	0.5	0.016	0.016	0.03	0.03	0.03
Imipenem	1	0.25	0.25	0.25	0.25	0.25
Imipenem-clavulanic acid	0.5	0.13	0.13	0.25	0.25	0.13
Ertapenem	32	0.016	≤0.008	0.016	0.03	0.016
Ertapenem-clavulanic acid	8	NT	NT	NT	NT	NT
Faropenem	4	1	0.5	0.5	1	0.5
Faropenem-clavulanic acid	4	0.5	0.5	0.5	0.5	0.25

[a] The β-lactamase inhibitors clavulanic acid and tazobactam were used at a fixed concentration of 4 μg/ml.
[b] R^+, transconjugant; R^-, recipient; NT, not tested.

CTX-M-15 producer isolated at the same hospital) were amplified with primers containing restriction sites (CTX-M-15-EcoRI-V, 5′-CGGAATTCAGCAAAGATGAAATC-3′, and CTX-M-15-BamHI-R, 5′-CAGGATCCTGAGTTTCCCCATTC-3′), digested with EcoRI and BamHI, ligated in the vector pBC, and expressed in *Escherichia coli* DH5α. The correctness of the cloned *bla* genes was confirmed by sequencing.

The pIs of the enzymes were compared by running sonicated cell extracts of CTX-M-71- and CTX-M-15-producing transformants on an IEF gel side by side or as a mixture of both. No difference between the pIs of CTX-M-71 and CTX-M-15 was detectable. The CTX-M-15-producing transformant showed higher MICs for cefotaxime (>16 times), ceftazidime, aztreonam (8 times), and cefepime (4 times) than the $bla_{\text{CTX-M-71}}$-harboring transformant (Table 1).

The purification of CTX-M-71 from the transformant and the determination of kinetic parameters were carried out as described previously (18). The data for CTX-M-15, previously obtained using the same procedure, were taken from the work of Queenan et al. (18).

Kinetic parameters are shown in Table 2. In comparison to CTX-M-15, CTX-M-71 showed a decreased hydrolytic efficiency for cefotaxime, due to both the decreased affinity (higher K_m value) and the lower turnover rates (k_{cat} values) for this substrate. The decrease of the hydrolytic efficiency of CTX-M-71 for ceftazidime and cefepime was less pronounced

TABLE 2. Kinetic parameters of CTX-M-71 and CTX-M-15

Substrate	CTX-M-71			CTX-M-15		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
Cephaloridine	68 ± 5	18 ± 1	3.8	188 ± 14	39 ± 3	4.82
Benzylpenicillin	58 ± 0.6	4.9 ± 0.2	11.8	47 ± 0.7	8.7 ± 3	5.40
Amoxicillin	49 ± 0.5	5.2 ± 0.9	9.4	NT[c]	NT	NT
Piperacillin	27 ± 0.5	3.2 ± 0.2	8.4	NT	NT	NT
Cefepime	8.8 ± 0.4	85 ± 4	0.1	45 ± 10	180 ± 37	0.25
Ceftazidime	0.69 ± 0.1	180 ± 11	0.0038	4.4 ± 0.1	236 ± 18	0.0186
Cefotaxime	65 ± 2	130 ± 0.7	0.5	176 ± 6	26 ± 3.6	6.77
Aztreonam	0.84 ± 0.06	10 ± 1	0.084	NT	NT	NT
Meropenem[a]	≤0.07	ND[b]	ND	≤0.004	ND	ND
Imipenem[a]	≤0.07	ND	ND	≤0.17	ND	ND
Faropenem[a]	≤0.46	ND	ND	NT	NT	NT
Doripenem[a]	≤0.05	ND	ND	≤0.002	ND	ND

[a] Hydrolysis was very slow; $V_{\max}$ was estimated as two times the maximum hydrolysis rate observed when the enzyme amount was increased 20- to 40-fold compared to that used for cephaloridine.
[b] ND, not determined, as hydrolysis was too slow to determine K_m.
[c] NT, not tested.

and was caused by lower turnover rates, while the affinities remained rather similar. These data correlate well with the MICs of the CTX-M-71- and CTX-M-15-producing transformant strains.

Although the weak inactivation of meropenem could be detected by the bioassay for the CTX-M-71-producing wild-type, transconjugant, and transformant strains as well as the CTX-M-15-producing transformant, the hydrolysis of all carbapenems was too slow to accurately determine kinetic parameters.

Our results demonstrate that the Gly238Cys substitution in CTX-M-71 decreases the hydrolytic efficiencies for cefotaxime and, to a lesser extent, for ceftazidime and cefepime. This is in concordance with the results of Shimazu-Ibuka et al. (19, 20), who introduced a Gly238Cys substitution in Toho-1, thereby establishing a disulfide bond between the cysteine residues at positions 69 and 238. This mutation decreased the activity against cefotaxime, cefuzonam, ceftazidime, and aztreonam; however, it also led to a higher thermal stability of the enzyme. The loss of activity was explained by the decreased flexibility of β-strand 3, which forms one wall of the active site cavity and which was locked due to the disulfide bond.

There is another pair of CTX-M β-lactamases for which the only difference is the glycine-to-cysteine exchange at position 238, CTX-M-10 and CTX-M-34. However, their phenotypes have not been compared (15).

To screen for further $bla_{\text{CTX-M-71}}$-harboring strains among CTX-M producers, 60 additional isolates were subjected either to $bla_{\text{CTX-M}}$ sequencing ($n = 26$) or to a CTX-M-71-screening PCR ($n = 34$) using the oligonucleotide CTX-M-238C-R (5′-GTGCCATAGCCACAG-3′), designed to discriminate the G-to-T nucleotide exchange at position 721. These 60 strains were recovered between 2001 and 2003 from seven hospitals in three Bulgarian towns (among them nine isolates from the Alexandrovska University Hospital) and comprised seven enterobacterial species. No further CTX-M-71 producer was detected. All other isolates produced either CTX-M-3 or CTX-M-15. Presenting a selection disadvantage, the decreased hydrolytic efficiency of CTX-M-71 might have been one of the reasons why no further CTX-M-71-producing isolates were found.

K. pneumoniae AH24-270 was resistant to ertapenem and showed decreased susceptibility to meropenem. The weak hydrolysis of meropenem in the bioassay and the effect of clavulanate on the MICs of meropenem and ertapenem suggested a contribution of CTX-M-71 to the resistance to carbapenems. However, clavulanate was not able to reduce the MICs of meropenem and ertapenem to a basic level; therefore, a second, nonenzymatic action seemed to be involved. The carbapenemase activity of CTX-M-71 seemed to be too marginal to influence the MICs of the transconjugant and transformant strains, which lacked additional resistance mechanisms. Since the CTX-M-15-producing transformant also showed weak meropenem hydrolysis in the bioassay, the minimal carbapenem activity is not attributable to the Gly238Cys exchange, which is in concordance with the results of Shimazu-Ibuka et al. (19), who found no influence of the Gly238Cys mutation on the carbapenemase activity of Toho-1. Therefore, it seems that the impaired susceptibility of *K. pneumoniae* AH24-270 to ertapenem and meropenem is caused by a combination of a nonenzymatic mechanism with a weak carbapenemase activity of CTX-M-71. This feature is not unique to CTX-M-71, as it has already been shown that the production of CTX-M ESBLs in porin-deficient *K. pneumoniae* may lead to ertapenem resistance (7, 9). Furthermore, the weak hydrolysis of ertapenem by CTX-M β-lactamases, initially detected by the synergy between ertapenem and clavulanate, has been described by Girlich et al. (8).

In conclusion, a *K. pneumoniae* isolate from Bulgaria producing a new CTX-M variant, CTX-M-71, characterized by the amino acid substitution Gly238Cys in comparison to CTX-M-15, was found. This exchange probably caused the formation of a disulfide bond, thereby decreasing the flexibility of β-strand 3 which led to impaired hydrolytic efficiency particularly for cefotaxime.

Nucleotide sequence accession number. The nucleotide sequence of $bla_{\text{CTX-M-71}}$ has been deposited in the GenBank database under accession number FJ815436.

We thank Wenchi Shang, Johnson & Johnson Pharmaceutical Research and Development, Raritan, NJ, for purification of the CTX-M-71 protein.

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