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Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum β-lactamase CTX-M-15 and of its structurally related β-lactamase CTX-M-3

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The extended-spectrum β-lactamase CTX-M-15 confers resistance to ceftazidime, unlike the majority of CTX-M-type enzymes. Kinetic parameters were determined from purified CTX-M-15 and CTX-M-3, which differ by the single amino acid substitution Asp-240 to Gly, according to the Ambler numbering of class A β-lactamases. Relative molecular masses of CTX-M-15 and CTX-M-3 were ~29 kDa and pI values were 8.9 and 8.4, respectively. CTX-M-15 had higher affinities for β-lactams (lower K_m values) than those of CTX-M-3 but catalytic efficiency (k_{cat}/K_m values) was variable depending on the β-lactam substrate. Only CTX-M-15 showed a measurable catalytic efficiency for ceftazidime. Clavulanic acid and tazobactam were good inhibitors of both enzymes. MICs of β-lactams for *Escherichia coli* reference strains expressing cloned β-lactamase genes in the same genetic background were similar except for ceftazidime. This work underlines the fact that some CTX-M enzymes may hydrolyse ceftazidime and thus confer resistance to this expanded-spectrum cephalosporin in Enterobacteriaceae.

Keywords: β-lactamase, CTX-M, expanded-spectrum β-lactamases

Introduction

In addition to the classical TEM and SHV enzymes, several plasmid-mediated Ambler class A extended-spectrum β-lactamases (ESBLs) have been reported. Among them, the CTX-M-type β-lactamases are currently spreading worldwide in Enterobacteriaceae.[1] The name 'CTX-M' refers to their potent hydrolytic activity for cefotaxime.[1,2] The CTX-M enzymes confer high-level resistance to cefotaxime, ceftriaxone and aztreonam, but have only marginal effects on MICs of ceftazidime for both wild-type and laboratory-derived strains of enterobacteria.[1] According to amino acid sequence data, they may be grouped in four clusters: CTX-M-1 (CTX-M-1, -3, -10, -11, -12, -15), CTX-M-2 (CTX-M-2, -4, -5, -6, -7, -20, Toho-1), CTX-M-8 and CTX-M-9 (CTX-M-9, -13, -14, -16, -18, -19 and Toho-2) (accession nos AJA16344 and 41346).[1–6]

Two novel point-mutant derivatives of CTX-M-9, CTX-M-16 and CTX-M-19, have been reported to hydrolyse ceftazidime significantly.[3,6] Additionally, we have reported recently the DNA sequence of another β-lactamase, CTX-M-15, from Indian enterobacterial isolates that were resistant to both cefotaxime and ceftazidime.[5] CTX-M-15 has a single amino acid change [Asp-240→Gly (Ambler numbering)][7] compared with CTX-M-3.[5] It has so far also been found in Japan (β-lactamase UOE-1; GenBank accession no. AY013478), Bulgaria[8] and Poland,[9] where CTX-M-3 is widespread.[10]

Since CTX-M-15-producing isolates had a significant degree of resistance to ceftazidime,[5] we have purified CTX-M-15 and CTX-M-3 and compared their kinetic parameters (kinetics of CTX-M-3 has not been studied before). Additionally, this report provides detailed kinetic data that are available only for a very few CTX-M-type enzymes.

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Materials and methods

Bacterial strains, cloning experiments and sequencing

CTX-M-15-producing *Escherichia coli* 2 was from India.[5] *Citrobacter freundii* isolate 2526/96, which was identified in Poland in 1996, was used as a $bla_{CTX\text{-}M\text{-}3}$-containing strain.[4] *E. coli* reference strain DH10B was used for cloning and expression experiments.[6] Cloning was carried out with PCR products generated with primers PROM+ (5′-TGCTCTGT-GGATAACTTGC-3′) and preCTX-M-3B (5′-CCGTTTC-CGCTATTACAAAC-3′) annealing to the 3′-end of insertion sequence IS*Ecp1* located upstream of $bla_{CTX\text{-}M\text{-}15}$ and downstream of $bla_{CTX\text{-}M\text{-}15/\text{-}3}$, respectively (accession no. AY0-44436).[3,4] Whole-cell DNA from *E. coli* 2 and *C. freundii* 2526/96 was used as template.[5] PCR amplimers were cloned into the *Srf*I site of the pPCRScript-Cam (SK+) plasmid (Stratagene Inc., La Jolla, CA, USA). Recombinant plasmids were transformed into electrocompetent *E. coli* DH10B cells and selected on Mueller–Hinton (MH) agar plates containing 100 mg/L ampicillin and 30 mg/L chloramphenicol. Sequencing of inserts of recombinant plasmids was carried out as described previously.[6]

Susceptibility testing

MICs of selected β-lactams were determined by the agar dilution technique on MH agar plates as described previously,[6] and interpreted according to the NCCLS guidelines.[11]

Biochemical analysis of CTX-M-15 and CTX-M-3

Cultures of *E. coli* DH10B with plasmids pCTX-M-15 and pCTX-M-3 were grown overnight at 37°C in 4 L of trypticase soy broth containing ampicillin (100 mg/L) and chloramphenicol (30 mg/L). β-Lactamase extracts were obtained using purification steps with a Q-Sepharose column, then an S-Sepharose column followed by elution at 50 mM NaCl, as described previously.[6] β-Lactamase-positive fractions were pooled and dialysed against 50 mM phosphate buffer (pH 7), and subsequently concentrated 10-fold with Centrisart-C30 microcentrifuge filters (Sartorius, Goettingen, Germany).[6]

Analytical isoelectric focusing (IEF) using an ampholine-containing polyacrylamide gel and purity of the enzymes and relative molecular masses estimated by SDS–PAGE analysis were carried out as reported previously.[6]

Purified β-lactamases were then used for kinetic measurements at 30°C in 100 mM sodium phosphate buffer (pH 7.0). The initial rates of hydrolysis were determined with an ULTROSPEC 2000 UV spectrophotometer (Amersham Pharmacia Biotech), as described previously.[6] The 50% inhibitory concentrations (IC_{50} values) were determined as reported previously.[6] Specific activities of the purified β-lactamases were evaluated as previously reported; one unit of enzyme activity was defined as the activity that hydrolysed 100 μmol of cefalothin per minute.[6]

Results and discussion

Recombinant plasmids and susceptibility testing

The DNA inserts of the two recombinant plasmids pCTX-M-15 and pCTX-M-3 were sequenced, confirming that they contained the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}3}$ genes, respectively. The 3′-end of IS*Ecp1* was located 48 and 128 bp upstream of the start codon of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}3}$, respectively (data not shown), indicating that the surrounding sequences of these two $bla_{CTX\text{-}M}$ genes were different.

E. coli DH10B that harboured pCTX-M-15 and pCTX-M-3 demonstrated a typical inhibitor-susceptible ESBL-mediated resistance profile (Table 1). MICs of β-lactams for *E. coli* DH10B (pCTX-M-15) mirrored those for *E. coli* DH10B (pCTX-M-3) except for ceftazidime; the MIC of ceftazidime for the CTX-M-15 producer was significantly higher than that for the CTX-M-3 producer.

Biochemical analysis of CTX-M-15 and CTX-M-3

The specific activities of purified β-lactamases CTX-M-15 and CTX-M-3 were 185 and 138 mU/mg of protein, respectively, with a 50-fold purification factor in both cases. Their purification level was ~90% (data not shown). IEF analysis identified pI values for CTX-M-15 and CTX-M-3 of 8.9 and 8.4, respectively. The relative molecular masses of CTX-M-15 and CTX-M-3, determined by SDS–PAGE analysis, were ~29 kDa (data not shown).

The glycine residue in position 240 in CTX-M-15 provided lower hydrolytic activity (lower k_{cat} values) for penicillins compared with CTX-M-3, as found for CTX-M-16 and CTX-M-9, which differ by the same amino acid substitution in position 240.[6] The overall hydrolytic activity of CTX-M-15 against cephalosporins was not higher than that of CTX-M-3, depending on the cephalosporin molecule.

CTX-M-15 had higher affinities (low K_m) than CTX-M-3 for all the β-lactams studied except for cefepime. This was particularly true for aztreonam, as found for CTX-M-16 when compared with CTX-M-9.[3]

In general, CTX-M-15 and CTX-M-3 had strong catalytic efficiency (high k_{cat}/K_m) against benzylpenicillin, piperacillin, cefotaxime and ceftriaxone (Table 2), as reported for other CTX-M-type enzymes such as CTX-M-16 and CTX-M-18.[3,6] The comparison of catalytic efficiencies of CTX-M-15 with those of CTX-M-3 revealed that cefuroxime and benzylpenicillin, respectively, were the best substrates for the two enzymes. The catalytic efficiencies of CTX-M-15 and

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Table 1. MICs of β-lactams for *E. coli* DH10B alone or harbouring recombinant plasmids pCTX-M-15 and pCTX-M-3 expressing CTX-M-15 and CTX-M-3, respectively

	MIC (mg/L)		
β-Lactam(s)[a]	*E. coli* DH10B (pCTX-M-15)	*E. coli* DH10B (pCTX-M-3)	*E. coli* DH10B
Amoxicillin	>512	>512	2
Co-amoxiclav	32	128	2
Ticarcillin	>512	>512	1
Ticarcillin + CLA	32	64	1
Piperacillin	>512	512	1
Piperacillin + TZB	4	2	1
Cefalothin	>512	>512	2
Cefuroxime	>512	>512	2
Cefotaxime	512	512	<0.06
Cefotaxime + CLA	2	2	<0.06
Cefotaxime + TZB	0.5	1	<0.06
Ceftazidime	256	32	<0.06
Ceftazidime + CLA	2	2	<0.06
Ceftazidime + TZB	2	2	<0.06
Ceftriaxone	>512	>512	<0.06
Cefepime	64	128	<0.06
Cefpirome	512	512	<0.06
Cefoxitin	4	2	2
Moxalactam	1	0.5	0.12
Aztreonam	64	128	0.06
Imipenem	0.25	0.25	0.12

[a]CLA, clavulanic acid at a fixed concentration of 2 mg/L; TZB, tazobactam at a fixed concentration of 4 mg/L.

Table 2. Steady-state kinetic parameters of purified CTX-M-15 and CTX-M-3 β-lactamases

	CTX-M-15			CTX-M-3		
Substrate	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	40	10	4	270	2.5	110
Amoxicillin	20	38	0.5	160	185	1
Ticarcillin	2	5	0.5	40	29	1
Piperacillin	35	13	3	180	66	3
Cefalothin	35	43	0.5	2800	96	30
Cephaloridine	130	83	1.5	130	300	0.5
Cefuroxime	70	13	5	3	49	0.07
Cefoxitin	<0.01	ND	ND	<0.01	ND	ND
Ceftazidime	2	1760	0.001	<0.01	>3000	ND
Ceftriaxone	135	37	3.5	30	58	0.5
Cefotaxime	150	54	3	380	113	3.5
Cefepime	10	1075	0.01	0.2	170	0.001
Cefpirome	120	195	0.6	30	316	0.1
Imipenem	<0.01	ND	ND	<0.01	ND	ND
Aztreonam	1.5	11	0.1	190	188	1

Values are means of three independent measurements (standard deviations of the values were within 15%); ND, not determinable (the initial rate of hydrolysis was lower than 0.01 $\mu M^{-1} s^{-1}$).

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CTX-3 did not correlate perfectly with the MIC values for *E. coli* producing CTX-M-15 and CTX-M-3, possibly caused by high copy number (~100 copies) of the cloning vector, which may substantially increase the amount of enzymes present in the periplasmic space.

In the case of ceftazidime, higher MIC values for CTX-M-15 producer than that for CTX-M-3 producer could be explained by different kinetic parameters. CTX-M-15, but not CTX-M-3, demonstrated a detectable, although relatively low, catalytic activity against ceftazidime, along with a low affinity for this substrate (high K_m value). Similar observations had previously been reported for the two other ceftazidime-hydrolysing CTX-M-type enzymes, i.e. CTX-M-16 and CTX-M-19.[3–5]

The kinetic parameters of CTX-M-15 against ceftazidime may be explained by the glycine residue at position 240. This amino acid residue at position 240 is not conserved among class A β-lactamases.[7] Some amino acid residues in this position have been found to play a key role in the extended hydrolytic profile of several ESBLs. Amino acid residue Gly-240 is found in other ESBLs such as VEB-1, BES-1 and PER-1.[3,12] Conversely, in a previous study,[12] we have reported that the substitution Gly-240→Glu in PER-1 caused a reduction in affinity of the enzyme for aztreonam and decreased its catalytic efficiency against cefotaxime and ceftazidime.

CTX-M-15 and CTX-M-3 were similarly prone to inhibition by clavulanic acid (IC_{50} values 9 and 12 nM, respectively) and by tazobactam (IC_{50} values 2 and 6 nM, respectively). The relatively higher susceptibility to inhibition by tazobactam compared with clavulanic acid is a feature of CTX-M-type enzymes.[1]

Data presented in this work indicate further that detection of CTX-M-type ESBLs can no longer be based only on a resistance pattern that includes resistance to cefotaxime and susceptibility to ceftazidime. The role of clinical usage of ceftazidime should be evaluated for selection of novel ceftazidime-hydrolysing CTX-M-type enzymes that may occur through a single amino acid substitution. This is true especially for the CTX-M-1- and CTX-M-9-type β-lactamases, which are spread worldwide.[1–6,8,10]

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